

Institute of Health and Community Medicine

The Epidemiology of Severe Community-Acquired Viral Lower Respiratory Tract Infection in Children Aged 5 Years and Below in Sarawak

Chai May Yin

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The Epidemiology of Severe Community-Acquired Viral Lower Respiratory Tract Infection in Children Aged 5 Years and Below in Sarawak

Chai May Yin

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DECLARATION

I declare that the work in this thesis was carried out in accordance with the regulations of Universiti Malaysia Sarawak. It is original and is the result of my work, unless otherwise indicated or acknowledged as referenced work. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.

_____

Name of Student : Chai May Yin

Student ID No : 12010067

Date :

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ABSTRACT

The epidemiology of acute lower respiratory infections (ALRIs) in children younger than five years of age has not been explored in Borneo using molecular techniques. Understanding the burden of disease in Sarawak setting is important in order to improve strategies to reduce the overall morbidity and mortality of acute respiratory disease. A three years prospective study was conducted from November 2012 to October 2015 among children with ARTIs aged five years and below who were admitted into the High Dependency Unit and Intensive Care Unit at 3 major hospitals in Sarawak which included Sarawak General Hospital (SGH), Sibu Hospital and Bintulu Hospital. A Multiplex PCR/RT-PCR approach was used to detect 18 respiratory viruses. Nasal flocked swab (NS), nasopharyngeal aspirates (NPA) or endotracheal tube secretion (ETT) from eligible children were collected. A total of 1204 viruses were detected in 902 of the total 1165 (77%) hospital admission episodes. The most frequent detected viruses were RSV (n=434, 37.2%), followed by HAdV (n=339, 29.1%) and HRV/EV (n=200, 17.2%). Co-detections were common, occurring in 270/902 (30%) of ARTIs. The most prevalent co-detections were RSV-HAdV (n=90) followed by HAdV-HRV (n=60). Overlapping seasonal trends of RSV and HAdV were observed. HAdV activity was positively associated with temperature while HPIV positively associated with temperature and negatively associated with rainfall. Pneumonia remains the main leading cause of ALRI in Sarawak mainly caused by RSV and HAdV. Acute bronchiolitis was mainly associated with RSV while HAdV, HRV and HPIV was associated with acute exacerbation of bronchial asthma, newly diagnosed bronchial asthma and viral wheeze to a similar degree. In conclusion, this three years study provided an important profile of the epidemiology of ALRIs in hospitalized children in Borneo. RSV, HAdV and HRV were predominant viruses found. More effort should be

put into the development plan for other respiratory viruses such as RSV and HAdV rather than influenza virus alone. From a public health perspective, information on the prevalence and seasonality of respiratory viruses are crucial to be used as a basis to optimize the timing of interventions for future vaccination programs such as RSV vaccines.

Keywords: Epidemiology, acute lower respiratory infections, etiology, multiplex-PCR, children

Kajian Epidemiologi Infeksi Saluran Pernafasan Akut Bahagian Bawah pada Kanak-Kanak Berumur Lima Tahun ke Bawah di Sarawak

ABSTRAK

Epidemiologi berkaitan dengan infeksi saluran pernafasan akut (ISPA) bahagian bawah pada kanak-kanak berumur lima tahun ke bawah tidak banyak dieksploitasi di rantau Borneo dengan menggunakan teknik-teknik molekular. Dengan memahami beban penyakit ini di Sarawak, ia dapat mempertingkatkan strategi bagi mengurangkan kadar morbiditi dan mortaliti penyakit infeksi saluran pernafasan akut ini. Kajian prospektif selama tiga tahun ini dijalankan di kalangan kanak-kanak berusia lima tahun ke bawah bermula dari November 2012 hingga Oktober 2015. Kanak-kanak yang terlibat terdiri daripada mereka yang dihantar ke unit terapi di tiga hospital utama di Sarawak iaitu Hospital Umum Sarawak (SGH), Hospital Sibu dan Hospital Bintulu. Multiplex PCR/RT-PCR dijalankan untuk mengenalpasti 18 jenis virus respiratori. Swab nasal, aspirasi nasofarink atau sekresi tuib endotrakea dikumpul daripada kanak-kanak yang memenuhi kriteria kajian ini. Antara semua 1165 episod pendaftaran hospital, sebanyak 1204 virus dapat dikesan dalam 902 (77%) episod positif. Virus yang paling banyak dikesan adalah RSV (n=434, 37.2%), diikuti dengan HAdV (n=339, 29.1%) dan HRV/EV (n=200, 17.2%). Pengesanan lebih daripada dua jenis virus adalah biasa dalam kajian ini dan meliputi 270/902 (30%) daripada infeksi saluran pernafasan akut. Pasangan pengesanan bersama yang paling lazim adalah RSV-HAdV (n=90) diikuti oleh HAdV-HRV (n= 60). Pertindihan bagi musim RSV dan HAdV diperhatikan. Aktiviti HAdV berkorelasi positif dengan suhu manakala HPIV berkorelasi positif dengan suhu tetapi berkorelasi secara negatif dengan hujan. Pneumonia merupakan punca utama ISPA di Sarawak hasil infeksi terutamanya daripada RSV dan HAdV. Bronkiolitis akut adalah dikaitkan dengan RSV manakala HAdV, HRV

and HPIV pula dikaitkan dengan eksaserbasi asma akut, bronkial asma yang baru didiagnosis dan pernafasan termengah-mengah. Kesimpulannya, kajian selama tiga tahun ini memberi profil asas yang penting dalam epidemiologi ISPA pada kanak-kanak yang dimasukkan ke hospital di Borneo. RSV, HAdV dan HRV merupakan virus yang paling utama dijumpai melalui kajian ini. Lebih banyak usaha perlu dipertimbangkan ke dalam pelan pembangunan untuk virus respiratori yang lain seperti RSV dan HAdV dan bukannya hanya tertumpu pada virus influenza sahaja. Dari perspektif kesihatan awam, maklumat mengenai prevalen dan musim virus respiratori adalah penting untuk digunakan sebagai asas bagi mengoptimumkan intervensi program pemvaksinan pada masa hadapan seperti vaksin RSV.

Kata kunci: *Epidemiologi, infeksi saluran pernafasan akut, etiologi, multiplex-PCR, kanak-kanak*

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LIST OF ABBREVIATIONS

°C	degree Celcius
AAP	American Academy of Pediatrics
ACCP	American College of Chest Physicians
AIDS	Acquired Immune Deficiency Syndrome
ARTI	Acute respiratory tract infection
ASA	Acetylsalicylic Acid
BA	Buenos Aires
BAL	Bronchoalveolar Lavage
CAP	Community-Acquired Pneumonia
CDC	Centers for Disease Control and Prevention
COPD	Chronic Obstructive Pulmonary Disease
CPE	Cytopathic Effect
DFA	Direct Fluorescent Antibody
dsDNA	Double stranded deoxyribonucleic acid
E	Envelope Protein
EKC	Epidemic Keratoconjuctivitis
ELISA	Enzyme-Linked Immunosorbent Assay
ERS	European Respiratory Society
ETT	Endotracheal Tube
EV	Enterovirus
F	Fusion protein
FCM	Flow Cytometry
G	Glycoprotein
GBD	Global Disease Burden

HA	Hemagglutinin
HAART	Highly Active Antiretroviral Therapy
HAdV	Human Adenovirus
HBoV	Human bocavirus
HCoV	Human coronavirus
HE	Hemagglutinin Esterase
Hep-2	Human Epithelial Cell Line
Hib	*Haemophilic influenzae* type b
HIV	Human Immunodeficiency Virus
HMPV	Human metapneumovirus
HN	hemagglutinin-neuraminidase
HPAI	Highly Pathogenic Avian Influenza
HPIV	Human parainfluenza virus
HRV	Human rhinovirus
ICTV	International Committee on Taxonomy of Viruses
IFN	Interferon
IFV	Influenza virus
IgE	Immunoglobulin E
IRES	Internal Ribosomal Entry Site
ITRs	Inverted Terminal Repeats
kb	kilo bases
L	Large protein
LLC-MK2	Rhesus Monkey Kidney Cell
LTRIs	Lower respiratory tract infections
M	Matrix protein
MDCK	Madin-Darby Canine Kidney Cells

MERS-CoV	Middle East Respiratory Syndrome Coronavirus
MLP	Major Late Promoter
MOH	Ministry of Health
mPCR	multiplex-PCR
NA	Neuraminidase
NFS	Flocked-Nasopharyngeal Swab
NIH	National Institute of Health
nm	Nanometers
NP	Nucleoprotein
NPA	Nasopharyngeal Aspirates
NS	Nasopharyngeal Swab
NSP	non-structural protein
NSAIDs	Nonsteroidal Anti-inflammatory Drug
ORF	Open Reading Frame
P	Phosphoprotein
PCR	Polymerase Chain Reaction
PICU	Pediatric Intensive Care Unit
pTP	Terminal Protein Precursor
RD	Human Rhabdomyosarcoma Cell Lines
RDRP	RNA-dependent RNA polymerase
RNP	Ribonucleoprotein
RSV	Respiratory syncytial virus
RT-PCR	Reverse Transcription Polymerase Chain Reaction
S	Spike protein
SH	Small Hydrophobic protein
ssRNA	single stranded ribonucleic acid

URTIs	Upper respiratory tract infections
UTRs	Untranslated Regions
VA RNA	Virus-Associated RNA
Vero	African Green Monkey Kidney
VPg	viral priming protein
VTM	Viral Transport Medium
WHO	World Health Organization

CHAPTER 1

LITERATURE REVIEW

1.1 Overview of respiratory tract infection

Acute respiratory tract infections (ARTI) are a substantial public health problem globally. Previous estimates by Global Burden of Disease (GBD) found that lower respiratory tract infections (LRTI) attribute to about 2.74 million deaths worldwide in 2015, making it the fifth leading cause of death overall and the leading infectious cause of death in children younger than 5 years (Troeger *et al.*, 2017). Although the pathogens causing ARTI vary geographically and by season, viruses were documented as important contributors to the burden of hospitalized ARTI in young children aged 5 years and below (Kim *et al.*, 2013; Kwofie *et al.*, 2012; Rowlinson *et al.*, 2017). Viral respiratory tract infections are often associated with severe respiratory diseases such as bronchiolitis, exacerbation of asthma, or pneumonia in early life (Cilla *et al.*, 2008; Costa *et al.*, 2014a; Nascimento *et al.*, 2010). Pneumonia is the severe form of acute LRTI. According to UNICEF (2015), pneumonia is responsible for more deaths of children under 5 than any other infectious disease. It was reported that 1 in 6 childhood deaths were due to pneumonia in 2015. South Asia and sub-Saharan Africa bear the burden of more than half of the total number of pneumonia episodes worldwide among children under five (Wardlaw *et al.*, 2006). In Malaysia, pneumonia accounts for 3% among the five principal causes of death in 2016, whereas among those aged below five years, pneumonia contributed a 3.8% of death (Department of Statistics Malaysia, 2017).

1.2 Definition of ARTI

The respiratory tract may be divided into two parts: the upper respiratory tract and the lower respiratory tract as shown in Figure 1.1. The upper respiratory tract includes the nose and nasal passages, paranasal sinuses, the pharynx (nasopharynx, oropharynx and laryngopharynx), middle ear and the portion of the larynx above the vocal cords. The lower respiratory tract includes the portion of the larynx below the vocal cords, trachea, bronchi, bronchioles and lungs (Simoes *et al.*2006).

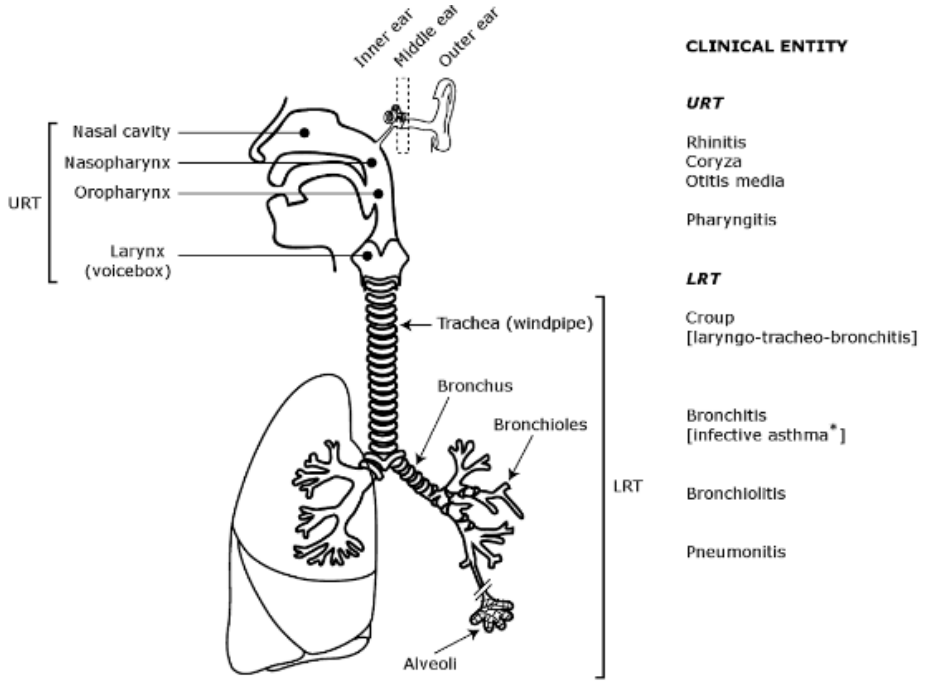


Figure 1.1: A Schematic Representation of The Human Respiratory Tract.
The schematic depicts the approximate locations of upper and lower respiratory tract (URT/LRT) diseases associated with infection by respiratory viruses.
(Mackay, 2007)

Infections of the respiratory tract are grouped according to their symptomatology and anatomic involvement (Figure 1.1). Thus upper respiratory tract infections are infections which occur proximal to the entrance of the airways starting from the nostrils and mouth to

the trachea, and including the paranasal sinuses and the middle ear which includes rhinitis, sinusitis, ear infections, acute pharyngitis/tonsillopharyngitis, epiglottitis and laryngitis/croup (Dasaraju & Liu, 1996).

ALRI are defined in the International Classification of Diseases as those infections that affect airways below the epiglottis which include acute bronchitis, acute bronchiolitis, pneumonia, lung infections or any combination among them, or with upper respiratory infections, including influenza (Lanata *et al.*, 2004). Severe LRTI hospitalization is defined as an LRTI requiring admission to the ICU or mechanical ventilation at any time during the LRTI hospitalization (Greenbaum *et al.*, 2014).

Several different clinical case definitions of ALRI have been proposed(Campbell *et al.*, 1989; Cherian *et al.*, 1988; Lanata *et al.*, 2004). The most widely used definition is based on the study by Shann and colleagues (1984) in Papua New Guinea who proposed a simple clinical method for the assessment of ALRI in children. Subsequently, the WHO then proposed that the three principal signs to determine acute respiratory infection severity include rapid breathing, chest in-drawing and inability to feed. From this, ALRI is classified into three categories according to severity as shown in Table 1.1.

Table 1.1: World Health Organization Guidelines for Assessment and management of ALRI

Diseases	Clinical Features	Management
Mild	Blocked or runny nose with cough, no tachypnoea (< 50 bpm), no chest indrawing, sore throat, ear discharge	Home treatment and supportive therapy
Moderate	Cough and tachypnoea but no chest indrawing	Home treatment with antimicrobials and supportive therapy
Severe	Cough and chest indrawing, cough and inability to feed or stridor at rest	Hospital referral

bpm = breaths per minute

Source: World Health Organization (WHO), 1990

1.3 Clinical manifestation of ARTIs

There are no clear-cut differences between the clinical presentation(s) of any viruses in the respiratory tract. For example, RSV is the most common cause of bronchiolitis worldwide; however, this clinical condition may also be caused by rhinoviruses, influenza viruses, human parainfluenza viruses or adenoviruses. Consequently, two patients with similar clinical illnesses must not be assumed to be infected by the same virus. This is particularly so in infants and young children. Conversely, the same virus may cause a range of clinical manifestations in different patients (Tregoning & Schwarze, 2010). The clinical manifestations of ARTIs are described in the following sections.

1.3.1 Upper respiratory tract infections

ARTI usually involves more than one anatomical location. URTI include rhinitis (common cold), sinusitis, otitis, sore throat (pharyngitis), tonsillitis, and laryngitis (inflammation of the voice box or larynx). URTIs are usually benign, transitory and self-limited, although epiglottitis and laryngotracheitis can be serious diseases in children and young infants (Dasaraju & Liu, 1996). URTIs are the most common of all communicable diseases. In infants, URTIs are often accompanied by fever and may lead to lethargy and poor feeding. Specific treatment is usually neither available nor required. However, analgesics or antipyretics (e.g., paracetamol) and in some cases, nasal decongestants may be helpful in reducing discomfort and symptoms which make feeding easier and allowing an adequate supply of oral fluids (Tregoning & Schwarze, 2010).

1.3.1.1 Rhinitis

Rhinitis (common cold) is irritation and inflammation of the lining of the nose. The main feature is a watery to mucoid, sometimes purulent nasal discharge (coryza). Rhinitis is categorized into three types: i) infectious rhinitis (includes acute and chronic bacterial infections; ii) nonallergic (vasomotor) rhinitis includes idiopathic, hormonal, atrophic, occupational and gustatory as well as drug-induced rhinitis; iii) allergic rhinitis (hay fever), triggered by pollen, mold, animal dander, dust and other inhaled allergens (Scadding *et al.*, 2008).

Rhinitis is commonly caused by viruses. Rhinoviruses are the most common pathogens. Coronaviruses, human parainfluenza viruses, respiratory syncytial virus, adenoviruses and influenza viruses have all been linked to the common cold syndrome. Classic symptoms of rhinitis clinically include runny nose, itching, nasal discharge and obstruction (congestion), sneezing, sore throat. Myalgia and headache may also be present. Complications are usually rare, but sinusitis and otitis media may follow (Dasaraju & Liu, 1996).

1.3.1.2 Sinusitis

Sinusitis is an acute inflammatory condition of one or more of the paranasal sinuses (Loebinger *et al.*, 2009). Acute sinusitis most often follows a common cold which is usually of viral etiology. Approximately 0.5% to 2% of viral sinusitis progress into bacterial infections and it is usually difficult to distinguish between bacterial and viral sinusitis. Typically, viral sinusitis resolves in 7 to 10 days, whereas bacterial sinusitis remains

persistent. Rhinovirus is the most common viral pathogen and is easily transmissible (Leung & Katial, 2008). Symptoms of sinusitis might include preceding common cold, purulent rhinorrhea, pain on bending, unilateral maxillary pain, and pain in teeth (Van Duijn *et al.*, 1992).

1.3.1.3 Otitis

Otitis which is the infection of ear can be caused by bacterial or viral pathogens (Massa *et al.*, 2009). Otitis externa is an infection involving the external auditory canal while otitis media denotes inflammation of the middle ear. Generally, itching, pain and tenderness of the ear lobe are present in otitis externa. The presence of purulent debris or obstruction of the ear canal by swelling may lead to the loss of hearing (Dasaraju & Liu, 1996). Otitis media typically presents as either acute otitis media (AOM), with symptoms including persistent severe earache (crying in the infant) accompanied by fever or irritability and short duration (Lieberthal *et al.*, 2013); or as otitis media with effusion (OME), which is often asymptomatic and characterised by accumulation of fluid in the middle ear (Rosenfeld *et al.*, 2004). Acute otitis media occurs most commonly in young children and is a common complication of URTI whose pathogenesis involves both viruses and bacteria (Pettigrew *et al.*, 2011). Common viruses that are frequently associated with otitis media include RSV, HRV, HAdV, HPIV and HCoV (Chonmaitree *et al.*, 2008). Bacteria that predominantly cause otitis media are *Streptococcus pneumoniae, Moraxella catarrhalis*, and non-typeable *Haemophilus influenzae* (Vergison, 2008).

1.3.1.4 Pharyngitis

Pharyngitis (sore throat) is an inflammation of the pharynx in which most cases are due to viral infections. Pharyngitis usually presents with a red, sore or 'scratchy' throat. Fever, reddening of the tonsils and pharynx, tonsillar exudates, enlarged and tender cervical lymph nodes, pain when swallowing (dysphagia) and headache may also present (Weber, 2014).

Various enteroviruses such as Type A coxsackieviruses can cause painful ulcerative pharyngitis (herpangina) in children most commonly aged 3-10 years old. Adenovirus and herpes simplex virus, although less common, also can cause severe pharyngitis (Bisno, 1996). The most important bacterial agent associated with acute pharyngitis and tonsillitis is *Streptococcus pyogenes* (group A β-hemolytic *Streptococcus* [GAS]), which is responsible for 15–30% of all cases of pharyngitis in children and for 10% in adults (Shulman *et al.*, 2012). Pharyngitis due to viruses is often associated with respiratory symptoms such as rhinorrhea, nasal congestion and cough. While GAS pharyngitis usually presents with the sudden onset of sore throat but do not have cough or nasal symptoms (Martin, 2015).

1.3.1.5 Croup

Croup refers to a number of respiratory illnesses that are manifested by varying degrees of stridor, barking cough, and hoarseness due to obstruction in the region of the larynx. It is derived from an Anglo-Saxon word meaning "to cry out", and is a common respiratory illness in children. The term croup has been used to describe a variety of upper respiratory conditions in children, including laryngitis, laryngotracheitis, laryngotracheobronchitis, bacterial tracheitis, or spasmodic croup (Miller *et al.*, 2013).

Young children are most susceptible to viral croup, which typically occurs in children 6 months to 3 years of age, with the mean age being 18 months. Boys are more likely to develop croup than girls. Parainfluenza virus type 1 is the most frequent cause of croup. Human parainfluenza viruses types 2 and 3 and influenza A also are major agents. Less common causes of croup are respiratory syncytial virus (RSV), influenza B virus, rhinoviruses, adenoviruses, enteroviruses, rubeola virus and *Mycoplasma pneumoniae* (Mustafa *et al.*, 2015).

Acute laryngitis usually begins as an upper respiratory infection, followed by dysphonia (hoarseness) and reduced vocal pitch or aphonia. Other symptoms may include odynophonia (pain when speaking), dysphagia (difficulty swallowing), odynophagia (pain when swallowing), sore throat, congestion, fatigue, and malaise (Mustafa *et al.*, 2015).

Patients with viral croup or laryngotracheobronchitis have a higher fever (38–39°C), including restlessness and more severe air hunger, compared to symptoms of laryngitis. Croup begins with a prodromal mild upper respiratory infection with coryza, nasal congestion, sore throat, and cough that lasts 2–3 days; this is followed by hoarseness and a harsh, brassy, "bark-like" cough. Respiratory stridor usually occurs at night, often awakening the child from sleep (Bjornson & Johnson, 2013).

1.3.1.6 Epiglottitis

Epiglottitis is an acute life-threatening bacterial or viral infection that results in swelling and inflammation of the epiglottis. Epiglottitis can acutely obstruct the trachea which can cause breathing problems, including stridor that can progressively worsen and may, ultimately resulting in a medical emergency (Tanner *et al.*, 2002). The syndrome of epiglottitis begins with the acute onset of fever, sore throat, hoarseness, drooling, dysphagia and progresses within a few hours to severe respiratory distress and prostration. The clinical course can be fulminant and fatal. The pharynx may be inflamed, and the epiglottis may appear 'cherry-red', thicken and swollen (Adil & Shah, 2015). Historically, *Haemophilus influenzae* type b (Hib) was the predominant organism (>90%) in pediatric epiglottitis cases. Prior to 1994, the disease was usually seen in children 2–7 years of age. The incidence of epiglottitis has markedly decreased which is due to the success of Hib vaccination of children (Shah *et al.*, 2004).

Croup and epiglottitis are often confused for each other as they share similar symptoms and signs including stridor, fever and respiratory distress. However, differentiation in early illness is possible by additional observation of coughing and absence of drooling in croup whereas there is drooling with absence of coughing in epiglottitis. Croup less likely causes severe airway obstruction and has a slow course, whereas untreated epiglottitis rapidly culminates in complete obstruction, shock and may be fatal (Tibballs & Watson, 2011).

1.3.2 Lower respiratory tract infections (LRTI)

Any infection below the location of the vocal cord is classified as LRTI. LRTI include conditions, which may or may not involve the parenchyma. Infections that doesn't involve the parenchyma include acute bronchitis, exacerbation of chronic bronchitis, bronchitis and bronchiolitis; whereas infections involving the parenchyma include pneumonia (Scaparrotta *et al.*, 2013). LRTI is more severe than URTI with increased likelihood of mortality (Berni *et al.*, 2017; Chang *et al.*, 2009; Gessner, 2011). For this reason, much of the focus of respiratory infection studies are on LRTI.

1.3.2.1 Acute bronchitis

Acute bronchitis is an acute or subacute cough illness lasting 2-3 weeks, with or without phlegm production. It is defined as inflammation of the bronchial respiratory mucosa, resulting in productive cough (O'Brien *et al.*, 1998). Bronchial epithelial injury is induced by infectious or non-infectious triggers, which cause an inflammatory response with consequent airway hyper-responsiveness and mucus production. Acute bronchitis is usually caused by a viral infection but can occasionally be complicated by secondary bacterial infections (Knutson & Braun, 2002).

Respiratory syncytial virus (RSV), parainfluenza virus and coronavirus are commonly found to be the main causative agents for acute bronchitis in patients younger than one year. In patients one to 10 years of age, parainfluenza virus, enterovirus, respiratory syncytial virus and rhinovirus predominate (Knutson & Braun, 2002). Other viral causes can be influenza A and B and human metapneumovirus. Less often, bacteria can also cause

acute bronchitis. Bacterial pathogens involved can be *Bordetella pertussis, Chlamydia pneumonia*e and *Mycoplasma pneumonia*e (Albert, 2010). Yeast and fungi which include *Blastomyces dermatitidis, Candida albicans, Candida tropicalis, Coccidioides immitis, Cryptococcus neoformans, Histoplasma capsulatum* might also trigger the cascade leading to acute bronchitis (Knutson & Braun, 2002). Cough is the most commonly observed symptom of acute bronchitis, beginning within 2 days of infection in 85% of patients, persisting in most of patients for less than 2 weeks. Other signs and symptoms may include dyspnea, wheezing, sputum production, chest pain, fever, hoarseness, malaise, rhonchi and rales, in varying degrees (Scaparrotta *et al.*, 2013).

Routine use of antibiotics is not recommended by the American College of Chest Physicians (ACCP) for patients with acute bronchitis, but they may be considered in certain situations: when pertussis is suspected as cough etiology, initiation of a macrolide is recommended as soon as possible to reduce transmission; however, antibiotics do not reduce duration of symptoms (Albert, 2010; Braman, 2006).

1.3.2.2 Infectious asthmatic bronchitis (wheezing)

Wheezy bronchitis, respiratory illness associated wheeze, and asthmatic bronchitis have been used in the past to describe episodic wheezing in infants and young children (Wilson, 1989). In epidemiological studies, by age of 6 years, almost half of the children were found to have experienced an episode of wheeze which is also the most common symptom seen in children with asthma (Brand *et al.*, 2008).

The European Respiratory Society (ERS) Task Force defines wheezing as a continuous high-pitched sound with musical quality emitting from the chest during

expiration and it is one of the forms of noisy breathing in preschool children (Brand *et al.*, 2008; Elphick *et al.*, 2001). Airway obstruction is a common symptom of lower respiratory viral infection but can also be triggered by other factors such as allergic inflammation or exercise. Children with wheezing run an increased risk of developing asthma and allergies, but not all children who wheeze have asthma (Martinez *et al.*, 1995).

Wheeze is often recognised clinically (or temporal pattern) as occurring with a viral respiratory infection (episodic viral wheeze) or being provoked by multiple triggers (multiple trigger wheeze) although the pattern may change over time. Other phenotypes can only be applied retrospectively (or in the long-term) and cannot be recognised or applied clinically (Brand *et al.*, 2008).

Episodic (viral) wheeze is defined as wheeze in separate episodes in response to viral infections, with the child being well between episodes that occur seasonally (Martinez *et al.*, 1995). Multiple-trigger wheeze is a wheezing that shows discrete flare-ups but also with symptoms of cough and wheeze between episodes, during sleep or with trigger factors such as allergens, vigorous activity, laughing or crying. The likelihood of asthma was noted to be much lower in children with episodic viral wheeze compared to children with multiple-trigger wheeze who wheezed during and in between episodes of viral infections which also reflects chronic airway inflammation (Brand *et al.*, 2008).

Wheezing illnesses are closely associated with viral infections in early childhood (Heymann *et al.*, 2004). There is a potentiality that some infants with recurrent wheeze in relation to respiratory viral infections in early life eventually develop asthma. This association has been best studied with RSV and HRV respiratory infections (Jackson *et al.*, 2008; Kusel *et al.*, 2007). For most infants, wheezing episodes with respiratory infections

diminish as the child ages, but for some individuals, early-life wheezing episodes may mark the beginning of asthma (Busse *et al.*, 2010).

Asthma has been defined as recurrent (≥ 3) episodes of wheezing dyspnea since birth. However, diagnosis of asthma is difficult in infants younger than 2 or 3 years (GINA, 2016) as wheezing, coughing and breathlessness are common in young children and can all be symptoms of conditions other than asthma (Brand *et al.*, 2008). According to Global Initiative for Asthma (GINA), asthma is a heterogeneous disease, usually characterized by chronic airway inflammation. It is defined by the history of respiratory symptoms such as wheeze, shortness of breath, chest tightness and cough that vary over time and in intensity, together with variable expiratory airflow limitation (GINA, 2016). Generally, asthma is identified by the presence of these symptoms, together with features of atopy and impaired lung function. Airway changes responsible for these symptoms are believed to be associated with airway hyper-responsiveness, atopy, and an inflammatory response leading to mucosal infiltration of inflammatory cells, oedema, mucus hypersecretion, and smooth muscle contraction (Landao, 1994).

Asthmatic bronchitis or bronchial asthma is a medical condition seen as a result of the combination of asthma and bronchitis. It is an allergic reaction in the bronchial tubes, which causes the bronchi to be more susceptible to inflammation. A person with asthma has an increased risk of developing bronchitis due to an increased sensitivity to airway irritation and inflammation. The tendency of the bronchial tubes to retract along with an increased production of mucous makes it difficult for a person to breathe and can lead to severe respiratory difficulties (Bierbaum & Heinzmann, 2007).

1.3.2.3 Acute exacerbation of bronchial asthma

According to the National Institute of Health (NIH) USA, National Asthma Education and Prevention Program (NAEPP, 2007) in the Expert Panel Report 3 (EPR-3), asthma exacerbations are acute or subacute episodes of progressively worsening in one or more typical asthma symptoms (shortness of breath, cough, wheezing, and chest tightness), characterized by decreases in expiratory airflow and objective measures of lung function (peak expiratory flow, PEF or FEV_1).

According to Global Initiative for Asthma (GINA), (2016), early symptoms of an acute exacerbation may include any of the following: an increase in wheezing and shortness of breath; an increase in coughing, especially nocturnal cough; lethargy or reduced exercise tolerance; impairment of daily activities, including feeding; and a poor response to short-acting β-agonist (SABA) medication. In general, an acute exacerbation of asthma is less likely to have fevers, and will often have a personal history of atopy or allergy, or a family history of asthma. It would be rare to diagnose a child under two with asthma at a first presentation to hospital with a wheeze.

Airflow obstruction in asthma exacerbations occurs because of concentric smooth muscle contraction, airway wall edema and luminal obstruction with mucus (Hegele, 2000). The main triggers of acute asthma exacerbations are numerous and include viruses, allergens (dust mite, pollen, animal dander), occupational exposures (grains, flours, cleaning agents, metals, irritants, woods), hormones (menstrual asthma), drugs (ASA, NSAIDs, betablockers), exercise, stress, and air pollutants. Common respiratory tract viruses, especially rhinoviruses, are the most common and important causes of exacerbation in both children and adults (Heymann *et al.*, 2004; Nicholson *et al.*, 1993). In 1995, Johnston and

his colleagues recruited 108 asthmatic children who were 9 to 11 years. In approximately 85% of children with acute asthma exacerbations, a respiratory virus can be detected, and nearly two-thirds of the detected infections were related to HRV.

Viral infection is likely to cause inflammation that primes the airway for enhanced responses to allergen exposure. It is likely that viral and allergic mechanisms act synergistically to increase the risk for acute exacerbation. Studies in children and adults demonstrated that allergic individuals who are exposed to allergens have the highest risk for virus-induced wheezing and asthma exacerbations. (Green *et al.*, 2002; Murray *et al.*, 2006).

Both viral infections and allergic inflammation can damage airway epithelium, and this could lead to synergistic effects. Viral infections compromise the barrier function of the airway epithelium, and could lead to enhanced absorption of allergens and/or irritants across the airway wall and increased allergic inflammation (Sakamoto *et al.*, 1984). Studies also showed that in damaged epithelium, HRV replication *in vitro* is greater and this implies that allergen-induced damages to airway epithelium could promote greater viral replication and more severe clinical illness (Jakiela *et al.*, 2008; Lopez-Souza *et al.*, 2004). Infection of airway epithelial cells with rhinovirus causes the release of proinflammatory cytokines and chemokines, as well as recruitment of inflammatory cells, especially neutrophils, lymphocytes, besides eosinophils and hence increases susceptibility to asthma exacerbation (Benoit & Holtzman, 2010).

1.3.2.4 Bronchiolitis

Bronchiolitis is the most common LRTI in infants aged 3 to 6 months. Infants and children with bronchiolitis often show upper and lower respiratory tract infection features together,

including rhinitis, cough, tachypnea, wheezing, crackles, nasal flaring and use of accessory muscles. Bronchiolitis of infancy is a clinically diagnosed respiratory condition presenting with breathing difficulties, cough, poor feeding and irritability, combined together with wheeze and/or crepitation on auscultation (SIGN, 2006). A consensus definition of bronchiolitis is "seasonal viral illness characterized by fever, nasal discharge, and dry, wheezy cough with fine inspiratory crackles and/or high-pitched expiratory wheeze on examination" (Lakhanpaul *et al.*, 2009).

Bronchiolitis is the main cause of hospitalization of infants younger than 1 year of age, with more than 80% of hospitalized children younger than 6 months. Children with underlying medical problems such as prematurity, cardiac disease or underlying respiratory disease are more prone to severe disease and so have higher rates of hospitalization. In preterm infants less than six months of age, admission rate with acute bronchiolitis is 6.9% more frequent admission to intensive care (Deshpande & Northern, 2003; Worrall, 2008).

As mentioned earlier, clinical signs and symptoms of bronchiolitis consist of rhinorrhea, cough, wheezing, tachypnea, and increased respiratory effort. Clinical conditions may deteriorate suddenly in the first 72 hours of the illness. Table 1.2 describes the clinical features of bronchiolitis.

Table 1.2: Clinical features of bronchiolitis

Clinical Features
1. Fever may be present, but high fever is uncommon and the absence of fever should not preclude the diagnosis
2. Nasal discharge often precedes other symptoms such as cough, tachypnea, respiratory distress and feeding difficulties
3. A dry, wheezy cough is another earliest symptom.
4. Increased respiratory rate is an important symptom
5. Feeding difficulties due to dyspnea
6. Dyspnea and subcostal, intercostal and supraclavicular recessions are common features, with the chest visibly hyperinflated in bronchiolitis
7. Fine inspiratory crackles in all lung fields are common
8. High-pitched expiratory wheeze is common but not always observed
9. Apnea can be the presenting feature of bronchiolitis, especially in the very young and in premature or low birth weight infants.

(Source: SIGN, 2006)

RSV is the commonest etiology of bronchiolitis which accounts for 70%-75% of the cases. This burden of disease is high, with around 70% of infants infected with RSV in their first year of life and 22% develop symptomatic disease (Glezen *et al.*, 1986; Worrall, 2008). The other viruses which can cause bronchiolitis included parainfluenza virus, adenovirus, rhinovirus, HMPV, coronavirus, and bocavirus (Miller *et al.*, 2013). HMPV accounts for 3% to 19% of bronchiolitis cases, with a clinical course similar of RSV (Zorc & Hall, 2010).

Bronchiolitis have long term effects on respiratory physiology and is linked with recurrent wheeze and asthma in later life. Based on certain research evidence, epidemiologic data suggest that early RSV bronchiolitis predisposes patients to recurrent wheezing and asthma. Sigurs and colleagues (2005) demonstrated that bronchiolitis in infancy was associated with recurrent wheeze/asthma at 7 and 13 years of age. Another study by Stein and collegues (1999) showed that RSV-related bronchiolitis in early childhood are an independent risk factor for subsequent development of wheezing up to age 11 years.

1.3.2.5 Pneumonia

Of all clinical presentations of ARTI, pneumonia is most associated with mortality globally. According to WHO estimates, 450 million cases of pneumonia are recorded annually with approximately 4 million of deaths from this illness. The highest incidences occur in children under 5 years and in adults older than 75 years (Rudan *et al.*, 2013; WHO, 2008). About 150 million children worldwide are affected by pneumonia yearly, of which 7-13% typically require hospital admission (Bhutta, 2007). Recently, UNICEF reported that pneumonia killed an estimated 663,000 children under the age of five in 2015, accounting for 16% of a total of 5.1 million deaths of children under five years old (Figure 1.2). According to UNICEF global databases published in 2015, pneumonia deaths among children aged 1-59 months in Malaysia reached 9.7% (UNICEF, 2015).

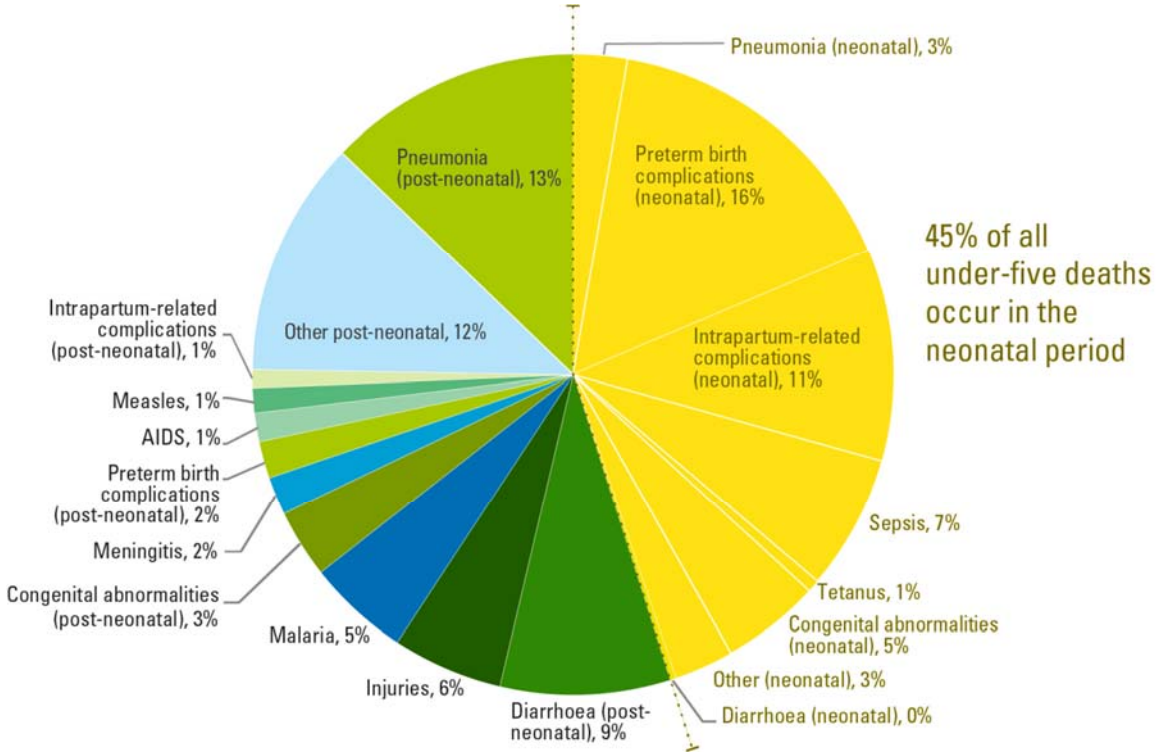


Figure 1.2: Global distribution of deaths among children under age five by cause, 2015 (Source: UNICEF, 2016)

Pneumonia is defined as an acute inflammation of the parenchyma of the lower respiratory tract, but this definition varies according to the organization, institution or health care setting. Clinically, URTI usually precedes pneumonia. Symptoms of pneumonia include fever, rigors, malaise, cough and dyspnoea (Wardlaw *et al.*, 2006). Clinical signs include chest in-drawing, dullness to chest percussion, crackles and/or wheeze on auscultation (Scott *et al.*, 2008). In contrast to bronchiolitis, chest radiography which shows consolidation, infiltrates, effusion, atelectasis and peribronchial thickening is in line with the WHO guidelines in diagnosing pneumonia (Cherian *et al.*, 2005). In general, the WHO definition of clinical pneumonia captures a broad spectrum of pediatric respiratory diseases, with (a) increased respiratory rate (Rate ≥ 60/min if age <2 months, ≥ 50/ min if age 2–11 months

and ≥ 40/ min if age 12–59 months); (b) lower chest wall in drawing (severe pneumonia); or (c) cyanosis and/or inability to feed or drink (very severe pneumonia)(WHO, 1991).

Pneumonia can be divided into community-acquired pneumonia (CAP) and hospital-associated pneumonia. The CAP was defined as pneumonia with onset prior to or less than 72 hours following admission to hospital, while the hospital-associated pneumonia was defined as pneumonia with onset 48 hours after admission (Puumalainen *et al.*, 2008; Scott *et al.*, 2012). The focus of this study will be of those related to CAP.

The vast majority of CAP may be caused by a wide variety of microbes, including many viruses, "typical" bacteria, atypical bacteria and fungi (Cevey-Macherel *et al.*, 2009; Ruuskanen *et al.*, 2011). With the advancement of diagnostic tests, pathogens are identified in 42% to 85% of children with community-acquired pneumonia. Viral CAP has been recognized as a frequent entity among children. In some studies, viral etiologies have been documented in up to 80% of episodes of CAP in children under 2 years old (Bradley *et al.*, 2011).

In a prospective study of children aged 2-59-months-old hospitalized with radiographically confirmed CAP in Brazil between September 2003 to May 2005, causative agent was detected in 86.2% of the 152 patients, with evidence of viral (46.4%), bacterial (14.4%) and mixed bacterial-viral (25.4%). Besides, in their study by comparing the frequency of bacterial and viral infections among children with CAP hospitalized across distinct severity categories, they concluded that respiratory viral infections were detected in a reasonable proportion of cases in each severity subgroup while bacterial infection, particularly pneumococcal infection, was more likely among severe/very severe cases (Nascimento-Carvalho *et al.*, 2016)

The distribution of pathogens varies with age and clinical setting. Table 1.3 shows the common causes of CAP in children. *Streptococcus pneumoniae, Haemophilus influenzae, Staphylococcus aureus* and RSV are considered to be the most important respiratory pathogens detected especially in areas without adequate pneumococcal and *H. influenzae* type b (Hib) vaccine coverage particularly in the developing world.

Table 1.3: Common causes of CAP in children

Bacteria	**Viruses**
Streptococcus pneumoniae	Respiratory syncytial virus
*Haemophilus influenza*e	Human metapneumovirus
Staphylococcus aureus	Parainfluenza virus types 1 and 3
Mycobacterium tuberculosis	Adenovirus
Atypical bacteria	Influenza A or B
Mycoplasma pneumoniae	Rhinovirus
Chlampophila pneumoniae	Human bocavirus
The predominant pathogens vary for children of different age groups: • < 2 months: Gram-negative bacteria, Group B streptococcus, *S. aureus, C. trachomatis*, viruses (RSV, Adenovirus, influenza and parainfluenza) • 2 months-5years: *S. pneumoniae, H. influenzae, S. aureus,* viruses. • 5 years: similar pathogens as 2 months-5 years age group; in addition *M. pneumoniae, C. pneumoniae*	

Source: Adapted from (Scaparrotta *et al.*, 2013)

Streptococcus pneumoniae is the predominant cause of bacterial pneumonia in children of all ages and is found in 30-40 % of cases as a single or co-pathogen (Ostapchuk *et al.*, 2004). Although *Staphylococcus aureus* is not a common cause of pediatric pneumonia, it has been increasingly encountered in communities where methicillin-resistant *Staphylococcus aureus* (MRSA) is prevalent. *Haemophilus influenzae type b* has almost disappeared because of vaccination. The overall incidence of pneumonia decreases with age, but it has been reported that the proportion of cases from atypical bacterial pathogens may increase among older children. *Mycoplasma pneumoniae* and *Chlamydophila pneumoniae* are more common causes of pneumonia among school-age children (Bradley *et al.*, 2011); in fact *Mycoplasma* infections have been common from the age of 5 years and

Chlamydophila infections from the age of 10 years onwards (Don *et al.*, 2010). Additionally, *Mycobacterium tuberculosis* has been identified in a proportion of acute pneumonia and can be an important cause of severe illness and death in children, especially in areas with high HIV infection prevalence (Oliwa *et al.*, 2015).

Fungal pneumonia in children is a rare condition, and is often seen in individuals with compromised immune system like AIDS. Pneumonia due to opportunistic fungal infections with *Pneumocystis jirovecii* is also frequent in HIV endemic areas. Other fungal agents that cause pneumonia in children are *Histoplasma capsulatum, Cryptococcus neoformans, Blastomyces* and *Coccidioides immitis* (Graham, 2005).

Severe forms of respiratory viral infections are rare in children but may lead to life-threatening conditions, such as severe pneumonia necessitating pediatric intensive care unit (PICU) admission and occasionally resulting in death (Gimenez *et al.*, 2007; Hon & Leung, 2009). Viruses may act as sole pathogens in pediatric CAP or predispose to bacterial pneumonia (Ampofo *et al.*, 2008). Respiratory viruses, especially respiratory syncytial virus (RSV) and rhinoviruses, either alone or in combination with bacteria, have been the most common aetiological agents of CAP in children under 2 years old (Don *et al.*, 2010) .

The ability to differentiate viral from bacterial pneumonia could have important management implications. There is increasing evidence that several different causal microorganisms may elicit different inflammatory responses, and levels of several markers such as white blood cells (WBC), C-reactive protein (CRP) and procalcitonin (PCT) are associated with different etiological patterns (Krüger *et al.*, 2009; Menéndez *et al.*, 2012). Clinical signs and symptoms of bacterial and viral pneumonia are highly variable and overlap, and there is no clinical or radiologic algorithm that exists to discern clearly the cause

of pneumonia. Differentiation based on biomarkers have been made by several researchers, and it is now well established that CRP and, especially, PCT showed higher levels in bacterial than in viral pneumonia (Hoshina *et al.*, 2014; Niederman, 2008). Table 1.4 depicts general variables used to distinguish viral from bacterial pneumonia. However, it is noted that the role of mixed bacterial-viral pneumonia (virus + bacteria) in CAP which accounted for approximately one-third of the cases has been described in recent years. A study done by Bello and colleague in 2014 found that high CRP level may be useful for clinicians to suspect mixed CAP.

Table 1.4: Variables used to distinguish viral from bacterial pneumonia

	Suggests viral cause	**Suggests bacterial cause**
Age	**Younger than 5 years**	**Adults**
Epidemic situation	Ongoing viral epidemic	-
History of illness	Slow onset	Rapid onset
Biomarkers		
Total white-blood cell count	< 10 x 10^6 cells per L	> 15 x 10^6 cells per L
C-reactive protein concentration in serum	< 20 mg/L	> 60 mg/L
Procalcitonin concentration in serum	< 0-1 µg/L	> 0.5 µg/L
Chest radiograph findings	Sole interstitial infiltrates, bilaterally	Lobar alveolar infiltrates
Response to antibiotic treatment	Slow or non-responsive	Rapid

(Source: Ruuskanen *et al.*, 2011)

1.4 Causative pathogens of acute respiratory infections

As mentioned in previous sections, there are a variety of respiratory pathogens including viral and bacterial which can cause ARTI. Although some causative agents have already been alluded to when describing the clinical presentation of ARTI, this section looks to highlight the causative pathogens of ARTI. Table 1.5 listed those common agents of respiratory infections.

Our study focused on the viral epidemiology of ARTI. In the past, there were more researches done on bacteria as bacteria were much more susceptible to culturing techniques. Viral pathogens are more difficult to detect and culture leading to late discovery of pathogens. Bacterial and fungal pathogens that are frequently discovered in childhood ARTI will be briefly described below but may not be mentioned again in this study due to its viral focus.

Table 1.5: Common agents of respiratory infections

Clinical illness	**Bacteria**	**Viruses**	**Fungi**	**Other**
Common cold (rhinitis, coryza)	Rare	- Rhinovirus - Coronavirus - Parainfluenza viruses - Adenoviruses - Respiratory syncytial virus - Influenza viruses	Rare	Rare
Pharyngitis and tonsillitis	- Group A β-hemolytic streptococci - *Corynebacterium diphtheriae* - *Neisseria gonorrhoeae* - *Mycoplasma pneumoniae* - *Mycoplasma hominis* (type 1)	- Adenoviruses - Coxsackieviruses A - Influenza viruses - Rhinovirus - Coronavirus - Parainfluenza virus - Epstein-Barr virus - Cytomegalovirus - Herpes simplex virus	*Candida albicans*	Rare

Table 1.5 continued

Clinical illness	Bacteria	Viruses	Fungi	Other
Epiglottitis and laryngotracheitis (croup)	- *Haemophilus influenzae* type b - *Corynebacterium diphtheriae*	- Respiratory syncytial virus - Parainfluenza virus	Rare	Rare
Bronchitis and bronchiolitis	- *Haemophilus influenzae* - *Streptococcus pneumoniae* - *Mycoplasma pneumoniae*	- Parainfluenza virus - Respiratory syncytial virus - Adenovirus - Herpes simplex virus	Rare	Rare
Pneumonia	- *Streptococcus pneumoniae* - *Staphylococcus aureus* - *Streptococcus pyogenes* - *Haemophilus influenzae* - *Klebsiella pneumoniae* - *Escherichia coli* - *Pseudomonas aeroginosa* - *Mycoplama pneumoniae* - *Legionella spp* - Anaerobic bacteria - *Mycobacterium tuberculosis* and other *Mycoplasma spp* - *Coxiella burnetii* - *Chlamydophila psittaci* - *Chlamydophila trachomatis* - *Chlamydophila pneumoniae* - *B. pseudomallei* - *Acinetobacter sp.*	- Adenovirus - Parainfluenza virus - Respiratory syncytial virus - Influenza virus - Varicella-zoster virus - Measles virus - Cytomegalovirus - Herpes simplex virus - Hantavirus - Coronaviruses - Human metapneumovirus - Human bocavirus - MERS-CoV - SARS-CoV - HBoV - HMPV	- *Histoplasma capsulatum* - *Blastomyces dermitidis* - *Paracoccidioides brasiliensis* - *Coccidioides immitis* - *Candida albicans* - *Filobasidiella* (cryptococcus) *neoformans* - *Aspergillus fumigatus* and other *aspergillus* spp	

(Source: Adapted from Dasaraju & Liu, 1996)

1.4.1 Respiratory viruses

With the emergence of modern laboratory methods, an etiological agent can be detected in patient presenting with signs and symptoms of an acute respiratory tract infection. Many of the common respiratory viruses have been identified between the 1930s and 1950s, and their clinical and epidemiological significance has been well established. Recently, driven by the developments in molecular techniques, new or previously unrecognized viruses have been identified (Table 1.6).

Table 1.6: Newly recognized respiratory viruses from 1993-2013

Year	Virus	Publications
1995	Sin Nombre virus	(Chizhikov *et al.*, 1995)
1998	Influenza A H5N1	(Subbarao, 1998)
1999	Influenza A H9N2	(Peiris *et al.*, 1999)
2001	Human metapneumovirus	(Bernadette *et al.*, 2002)
2002	SARS-CoV	(Kuiken *et al.*, 2003)
2004	Coronavirus NL63	(van der Hoek *et al.*, 2004)
2004	Influenza A H7N7	(Koopmans *et al.*, 2004)
2005	Human bocavirus	(Allander *et al.*, 2005)
2005	Coronavirus HKU1	(Woo *et al.*, 2005)
2007	Rhinovirus C	(Lau *et al.*, 2007)
2007	WU and KI polyomavirus	(Allander *et al.*, 2007a; Gaynor *et al.*, 2007)
2009	Influenza A H1N1	(Dawood *et al.*, 2009)
2012	MERS-CoV	(Zaki *et al.*, 2012)
2013	Influenza A H7N9	(Gao *et al.*, 2013)
2013	Influenza A H10N8	(To *et al.*, 2014)

(Source: Adapted from Al-Tawfiq *et al.* 2014)

Taxonomically, respiratory viruses can be divided into six families. Table 1.7 below summarized the viruses involved in this study which are more often associated with respiratory tract infections. Other viruses can also cause systemic disease with respiratory tract involvement as part of an overall disseminated disease process in patients who are immunocompetent or immunocompromised (e.g. cytomegalovirus).

Table 1.7: Summary of classification for viruses associated with respiratory tract infections

Virus	**Family**	**Genus**	**Serotypes or subtypes**	**Composition of virus**
Human rhinovirus (HRV)	*Picorna-viridae*	Enterovirus	> 102 serotypes phylogenetically divided into 3 groups A, B and C	Nonenveloped Positive sense-ssRNA virus, 7.2kb
Enterovirus 68		Enterovirus	EV-D68	Nonenveloped, Positive sense-ssRNA virus, 7.5kb
Influenza virus	*Orthomyxo-viridae*	*Orthomyxovirus*	Species A, B & C	Enveloped, negative sense-ssRNA, 13.5kb
Parainfluenza virus 1, 3 (HPIV)	*Paramyxo-viridae*	*Paramyxovirus*	Human human parainfluenza viruses types 1 and 3	Enveloped, Negative sense-ssRNA, 15-16 kb
HPIV 2, 4		*Rubulavirus*	Human human parainfluenza viruses types 2, 4a, and 4b	
Human respiratory syncytial virus (RSV)		*Pneumovirus*	2 subtypes (A & B)	Enveloped, Negative sense-ssRNA, 15kb
Human metapneumo-virus (HMPV)		*Metapneumo-virus*	2 subtypes (A & B)	Enveloped, Negative sense-ssRNA, 13kb
HCoV-229E, HCoV-NL63	*Corona-viridae*	*Coronavirus*	Group 1 (mammalian)	Enveloped, Positive sense ssRNA, 27-32kb
HCoV-OC43, SARS-CoV, HCoV-HKU1, MERS-CoV			Group 2 (mammalian)	
Human adenovirus (HAdV)	*Adenoviridae*	Mastadenovirus	> 51 Subgenera A to G	Nonenveloped, dsDNA, 26-48kb
Human bocavirus (HBoV)	*Parvoviridae*	*Bocavirus*	One phylogenetic group	Non-enveloped, Negative or positive sense, ssDNA, 4000-6000nt

1.4.1.1 Respiratory Syncytial Viruses (RSV)

Respiratory syncytial virus was first isolated in 1955 from adolescent monkeys with severe coryza illness and was initially called chimpanzee coryza agent. Soon thereafter, the virus was isolated from children with bronchiolitis and pneumonia and was recognized as the most common cause of annual winter epidemics of lower respiratory tract infections throughout the world. It was then renamed respiratory syncytial virus in 1957 as the virus had a proclivity for the respiratory tract and formed multinucleated giant cell syncytia in tissue cultures (Prober & Sullender, 1999).

1.4.1.1.1 Virus properties

RSV belongs to the family *Paramyxoviridae*, subfamily *Pneumovirinae.* RSV is an enveloped virus with a non-segmented, single stranded, negative-sense 15Kb RNA genome. The virus appears as an irregular spherical particle of 100 to 350mm in diameter when visualized with the electron microscope (Cane, 2001). The RSV genome includes 10 genes that encode for 11 proteins (Collins *et al.*, 2001). Figure 1.3 illustrates schematically the 11 RSV encoded proteins in which the M2 protein is actually divided into two proteins M2-1 and M2-2. A description of the proteins is shown in Table 1.8.

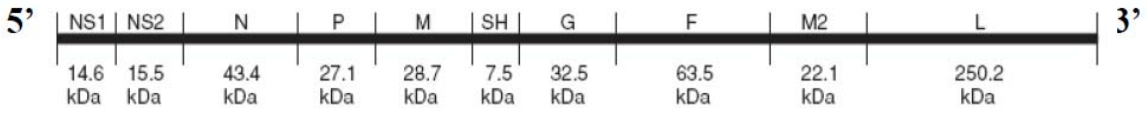


Figure 1.3: The RSV genome

Viral genes are shown above the line, while location and size of the viral protein are shown below the line (source: McNamara & Smyth, 2002).

Table 1.8: Characteristic of RSV Protein

Protein	**Molecular mass (Kilodaltons)**	**Functions**
Structural protein **Surface**		
Fusion (F)	68	Penetration; major protection antigen
Attachement (G)	90	Viral attachment; major protective antigen
Small Hydrophobic (SH)	4.8-30*	Unknown
Matrix		
Matrix (M)	28	Mediates attachment of nucleocapsid to envelope
Small envelope (M2)	22*	Transcriptional regulation; unique to pneumovirus
Nucleocapsid-associated		
Nucleoprotein (N, NP)	44	Major RNA-binding nucleocapsid protein
Phosphoprotein (P)	37	Major phosphorylated protein; RNA-dependent RNA polymerase activity
Large Polymerase Complex (L)	200	Large nucleocapsid-associated protein; major polymerase subunit; RNA-dependent RNA polymerase activity
Nonstructural protein		
Non-structural (NS1)	15.6*	Function unknown; unique to pneumoviruses
Non-structural (NS2)	14.7*	Function unknown; unique to pneumoviruses
* There are four glycosylated and non-glycosylated forms with molecular masses of 4.8, 7.5, 13 to 15 and 21 to 30KDa		

(Source: Hall, 2001)

RSV consists of three transmembrane glycoproteins: the attachment protein (G), the fusion glycoprotein (F) and the small hydrophobic protein (SH). Figure 1.4 shows the viral structures of RSV. The G protein is responsible for viral attachment to host cells, while the F protein promotes syncytial formation (McNamara & Smyth, 2002). Both F and G glycoproteins are the major targets of neutralizing antibodies (Gottschalk *et al.*, 1996). They are the main antigens responsible for inducing a neutralizing immune response and

resistance to infection. RSV infection into the host cell begins with the G protein binding to the host cell, followed by F protein-mediated fusion of the viral envelope into the cell membranes and penetration of the nucleocapsid complex into the cytoplasm. The F and G proteins stick from the virus envelope and are responsible for attachment (G-protein) and fusion (F-protein) to host cells (Gottschalk *et al*. 1996). Cane & Pringle (1995) highlighted that the attachment G protein shows the most genetic variability among all RSV proteins. SH is a small integral membrane protein whose function is unclear. It is phosphorylated by an MAPK p38-dependant typrosine kinase to achieve its normal cellular distribution in infected cells (Sidwell & Barnard, 2006).

RSV inner portion of the envelope interacts with the mature (M) protein, which directs the assembly of virions within the inner side of the host membrane from which the viral envelope is derived. Nucleocapsid which is found within the envelope is made up of the major nucleocapsid N-protein that binds to genomic RNA, a phosphoprotein (P), a transcription anti-terminator factor or transcriptase processivity factor (M2-1), and the large polymerase subunit (L). The N, P and L proteins are responsible for replication, while the M2-1 protein is required for efficient transcription. In addition, there are two non-structural protein referred to as NS1 and NS2 and a regulatory protein known as M2-2 (McNamara & Smyth, 2002).

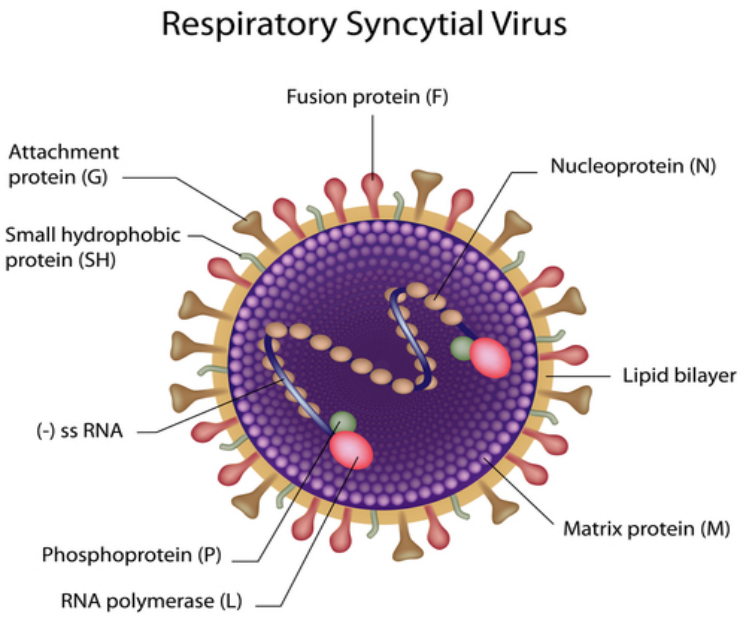


Figure 1.4: Structure of RSV (Source: Hall, 2001)

1.4.1.1.2 Antigenic and genetic diversity

RSV has a non-segmented RNA genome. Thus, it does not have the capacity for reassortment of genome segments. However, RSV has a quite mutable genome due to its dependence on an RNA polymerase that lacks the capacity of RNA proof-reading and editing (Sullender, 2000). RSV strains are separated into subgroups A and B based on antigenic and genetic variability. The main differences between RSV-A and RSV-B were found in the attachment glycoprotein G (Anderson *et al.*, 1985; Mufson *et al.*, 1985).

RSV A and B subgroups have been subdivided further into genotypes, by genetic analysis as follows:

1) RSV –A is divided into 12 genotypes. Ten of these genotypes are named GA1 to GA7, South Africa A1 (SAA1), NA1 and NA2 which were genetically close to GA2 strains

(Peret *et al.*, 2000; Shobugawa *et al.*, 2009; Venter *et al.*, 2001). The other two were novel genotypes named RSV-A ON1 which was first detected in November, 2010 in Ontario, Canada and the other possible new genotype RSV-A ON2 was reported in Rome, Italy in 2013 (Eshaghi *et al.*, 2012; Hirano *et al.*, 2014).

2) RSV-B is divided into 20 genotypes which include GB1-GB4 (Peret *et al.*, 1998), BA1-BA6 (Trento *et al.*, 2006), BA7-BA10 (Dapat *et al.*, 2010), SAB1-SAB3 (Venter *et al.*, 2001), SAB4 (Arnott *et al.*, 2011), URU1 and URU2 (Blanc *et al.*, 2005)

Both group A and group B strains circulate at the same time but group A usually dominates (Anderson *et al.*, 1985). The predominance of RSV-A over RSV-B viruses has been attributed to the higher variability among the RSV-A strains. Comparative data were reported in a review by Sullender (2000). In a comparison made among two of the group A viruses, the G proteins within the group shared 94% amino acid identity while there was 47% amino acid differences between group A and B G protein (Johnson *et al.*, 1987). In another study comparing the group B viruses, genetic similarity within the group was about 98%, whereas the group A and B G-protein gene sequences were quite divergent (44% amino acid differences) (Sullender *et al.*, 1990).

Venter *et al.*, (2001) studied RSV over four consecutive seasons (1997-2000) in South Africa showed that subgroup A revealed more variability and displacement of genotypes while subgroup B remained more consistent. Subgroup A showed a gradual build-up and then replacement of dominant genotypes, for example, GA5 (1997) was replaced by SAA1 (1998), which was then replaced by GA2 (1999). GA2 predominated for more than one season, increasing from 42% (1999) to 78% (2000). A study done by Parveen *et al.*, (2006) in India also proved that the level of genetic variability was higher among the

group A viruses (up to 14%) than among the group B viruses (up to 2%). Zhang *et al.* (2007) studied the variability of RSV in China also showed that group A prevailed over group B.

In Israel, the changes in the RSV subtype observed from 2008 were detected for both the GA and the BA subtypes (Hirsh *et al.*, 2014). The GA2 subtype was present in very high percentages, as observed in other geographic areas such as China and India. The continuous and predominant circulation of subtype GA2 demonstrate that this subtype is stable and has become epidemic in many countries. The dominant subtype of RSV-B was BA. This observation lies in agreement with several reports demonstrating that the BA subtype has become the most dominant subtype of RSV B globally (Goto-Sugai *et al.*, 2010; Parveen *et al.*, 2006; Pretorius *et al.*, 2013).

The relationship between clinical severity and RSV subgroups had been evaluated in several studies. In approximately half of the studies, RSV-A seemed to be associated with more severe clinical disease (Fletcher *et al.*, 1997; Gilca *et al.*, 2006; Hall *et al.*, 1990; Martinello *et al.*, 2002; McConnochie *et al.*, 1990; Papadopoulos *et al.*, 2004; Tran *et al.*, 2013; Walsh *et al.*, 1997) while other studies demonstrated no significant differences in clinical severity between the two subgroups (Brandenburg *et al.*, 2000; Fodha *et al.*, 2007; Gamino-Arroyo *et al.*, 2017; Kneyber *et al.*, 1996; McIntosh *et al.*, 1993; Tsutsumi *et al.*, 1991). In only two studies have group B infections been reported to cause more severe disease (Hornsleth *et al.*, 1998; Straliotto *et al.*, 2004). This inconsistency could be attributed to difference in study design and population, method of analysis, definition of disease severity and also the distribution of RSV subtypes.

In a comparison between the two groups, Oliveira *et al.* (2008) reported that group B strains tend to grow more slowly in tissue culture, produce fewer virus particles and lose

the characteristic of syncytial formation. This could reduce the ability to isolate the virus from clinical specimens. Besides that, group A viruses replicate to higher titers than the group B viruses in both cell culture and animals suggesting that RSV-A viruses are more virulent than the RSV-B viruses in humans. It has also been suggested that certain virus variants within group A are responsible for the disease severity and thus further complicating the comparisons of clinical severity (Fletcher *et al.*, 1997; McConnochie *et al.*, 1990; Walsh *et al.*, 1997). In addition, RSV disease severity is likely to be determined by an interplay between host and virus factors, including RSV load (Fodha *et al.*, 2007).

1.4.1.1.3 Epidemiology and seasonality

RSV is the most common pathogen leading to hospitalization in young children up to the age of five. Approximately two thirds of infants are infected with RSV within their first year and 90% have been infected by the age of two (Shobugawa *et al.*, 2009). The infection with RSV is a disease in both developing as well as developed countries. It was proven by many researchers that almost all children have been infected at least once with RSV (Dapat *et al.*, 2010; Reiche & Schweiger, 2009; Shobugawa *et al.*, 2009). McNamara & Smyth (2002) reported that by the age of 18 months, 87% of children have antibodies to RSV and by the age of 3 years, virtually all children are infected.

In most published studies, RSV outbreaks in area with temperate or Mediterranean climates in the southern hemisphere appear mainly during the cold winter months (Gilchrist *et al.*, 1994; Martin *et al.*, 1978). This might be due to crowding of susceptible individuals indoors in winter and the cooling of the nasal passages with concomitant decrease in respiratory defence. Most communities with temperate climates have a well-defined season

of 3 to 5 months usually starting in the Northern hemisphere in October or November and continuing until February or March. In the southern hemisphere, wintertime epidemics occur from May to September. Biennial change was observed from one season to the next whereby the subsequent season is milder or more severe than the preceding. However, this is not predictable and the differences may be more related to climatic changes (Light, 2012).

In tropical and subtropical climates with seasonal rainfall, the outbreaks of RSV were more associated with the rainy season rather than the cold season. They found that the peak of the RSV season occurred one to two months after the onset of the rains (Weber *et al.*, 1998). In Singapore, the seasonal trends of viral respiratory infections were reviewed by Chew and colleagues (1998). They reviewed the viral isolates from 1990 to 1994 and reported 3904 positive viral cultures. RSV was the most dominant pathogen detected with 72% of the total. In the same study, RSV showed significant annual seasonal trends with increase in cases between the months of March and August with a peak in May-June. The RSV trends were associated with higher environmental temperature, lower relative humidity and higher maximal day-to-day temperature variation. There was a definite season noted related to these climate features.

A larger study from Malaysia which included 5691 children less than 24 months hospitalized with LRTI between 1982 and 1997. The RSV season showed a seasonal pattern with peaks in the months of November, December and January. The rate of RSV infection appeared to correlate with the number of rain days and inversely with the monthly mean temperature (Chan *et al.*, 2002). In another 6-month study from June to December 2009 done by Etemadi and colleagues (2013a), a continuous activity of RSV was seen with a small peak in July, and then RSV activity started to increase from August to October with a relatively big peak in October. During the study period, both antigenic subgroups of A and B co-

circulates with predominance of subgroup B. However, this study only presents a small picture of the circulation pattern of RSV genotypes in Malaysia.

A more representative result required a longer study period. Khor and colleagues (2013) conducted a study from 1989 to 2011 in Kuala Lumpur, Malaysia showed that both RSV subgroups were also found co-circulating each year. RSV-A was present every year, while RSV-B was not detected in 6 years. RSV-A predominated in 12 out of 17 years while RSV-B predominated in 5 different years (1989, 1999, 2002, 2003 and 2009). In 1994–1999, the RSV-A genotypes GA2 and GA7 were most commonly detected in the study. These genotypes were gradually displaced by GA5, NA1, and NA2 in the 2000s. NA2 was the predominant genotype in 2000, 2004 and 2005, while NA1 has become strongly predominant. The BA genotypes replaced the predominant RSV-B genotype SAB3 from 2000 onwards. Besides that, they also observed a unique local cluster, BA12 in 2009.

Through the many studies conducted, it is clear that RSV infection is seasonal in the tropics and it is more common during the rainy season. During the rainy season, children tend to be kept indoors, and the resultant crowding may lead to the increase in incidence of RSV during this period. Another possibility is that high humidity may be conducive to viral survival by preventing drying and loss of infectivity of the virus. RSV is also known to be a labile virus, and does not survive well at high temperature, which may explain the relationship with cooler months (Shek & Lee, 2003; Welliver, 2007).

1.4.1.1.4 Clinical presentation

RSV was determined to be the leading cause of lower respiratory tract infections particularly in young infants. The severity of the disease is very diverse ranging from mild cold

symptoms to severe and life-threatening. It's the leading cause of pneumonia and bronchiolitis in infants. Children infected with RSV usually present with URTI symptoms on the onset, which may then worsen to LRTI, such as rhinitis (stuffy nose), cough and coryza (runny nose). The cough may be prolonged for several weeks with the acute illness lasts for about 5 – 10 days. The most common auscultatory signs are crackles and wheezes (Hall *et al.*, 2009).

In a study by Hall & Anderson (2013), hospitalized infants with RSV positive among those ≤ 1 year of age are frequently diagnosed with bronchiolitis while those older children 2 – 5 years of age was diagnosed more frequently with asthma and pneumonia. In developing countries, co-infection or secondary bacterial infection may further aggravate RSV disease (Madhi & Klugman, 2004). Up to 30–50 % of infants hospitalized with RSV lower respiratory infection have been reported for recurrent wheezing and long-term pulmonary sequelae (Sigurs *et al.*, 2010; Sly *et al.*, 2010; Stein & Martinez, 2010). However, whether RSV infection causes the predisposition to recurrent wheezing or a predisposition to recurrent wheezing increases the risk of RSV hospitalization still remains unclear (Stensballe *et al.*, 2009).

1.4.1.1.5 Treatment and prevention

For children who require hospitalization for RSV infection, supportive therapy is still the mainstay of care. Such therapy may include supplemental oxygen for hypoxemia, intravenous (IV) fluids for dehydrated or poorly feeding infants and mechanical ventilation (Cooper *et al.*, 2003). Bronchodilator therapy (albuterol, nebulized racemic epinephrine), systemic corticosteroids, and ribavirin have failed to show clear benefit in randomized controlled trials and are not currently recommended for use in many countries (Mitchell, 2009; Zorc & Hall, 2010).

Currently, there is still no licensed vaccine to prevent RSV infection. Extensive studies on vaccination strategies are still being conducted for RSV prevention, but only palivizumab (Synagis®) has been approved for use to a limited extent in United States among children who are at high risk of severe RSV disease – those born before 32 weeks gestation, or with significant cyanotic heart disease or chronic lung disease. Though not a vaccine, palivizumab (Synagis®) is an FDA-approved prescription injection of the neutralizing monoclonal antibody (mAb) that is given monthly to help protect high-risk infants from severe RSV disease throughout the RSV season (Higgins *et al.*, 2016; Turner *et al.*, 2014).

The success of RSV F mAb (palivizumab and motavizumab) which elicits functional antibody responses in passive immunization provides the rational for vaccine development. Antibodies to F protein are cross-reactive across RSV-A and RSV-B subtypes while antibodies to G protein are much less so. This led to the focus of RSV F-specific antibodies as a primary immunogen. Despite the challenges faced in vaccine development, a number of candidate vaccines are in preclinical or clinical development which encompasses five broad platforms. As shown in Figure 1.5, several live-attenuated, genetically engineered virus and

subunit-based vaccines are under clinical evaluation and many more vaccine candidates are in preclinical development (Higgins *et al.*, 2016).

Vaccine candidates for RSV prevention would certainly benefit public health by providing protection for those immunized besides decreasing spread to at-risk populations by improving herd immunity. Understanding the epidemiology of RSV is thus important to choosing a study population that is suited to efficient evaluation of candidate vaccines. We hope in future, advances in therapeutic and vaccine development could open new opportunities in control of RSV disease.

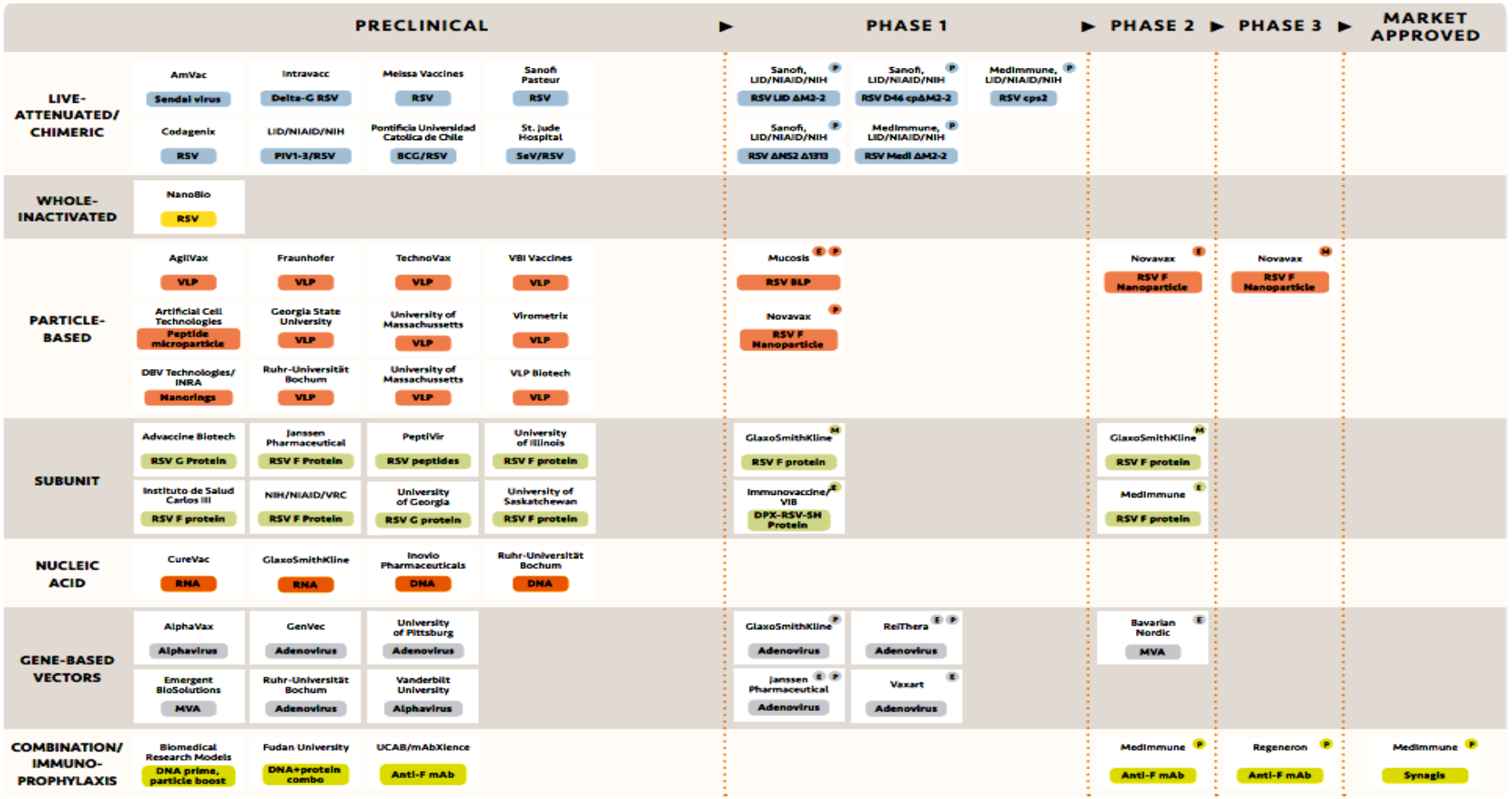


Figure 1.5: RSV vaccine development
(Source: PATH, 2016)
http://sites.path.org/vaccinedevelopment/respiratory-syncytial-virus-rsv/

1.4.1.2 Human metapneumovirus (HMPV)

In 2001, van den Hoogen and colleagues reported the discovery of a novel virus from children with respiratory tract illness in The Netherlands. HMPV has been classified in the *Metapneumovirus* genus of the *Paramyxoviridae* family (van den Hoogen *et al.*, 2001). Several lines of evidence suggested that HMPV was a common human respiratory pathogen and it has been detected in 4-16% in children under 5 years old with ARTIs which required hospitalization (Edwards *et al.*, 2013; Nicholson *et al.*, 2006; Panda *et al.*, 2014). HMPV resembles RSV with regard to disease signs and the ability to infect and cause disease in young infants as well as individuals of all ages (Boivin *et al.*, 2002).

1.4.1.2.1 Virus properties

The HMPV virion is pleomorphic in nature and its size varies from 150nm to 600nm (van den Hoogen *et al.*, 2001) (Figure 1.6). The genomic orientation of HMPV resembles other members of the *Paramyxoviridae* family. The genome organization of HMPV is quite similar to that of avian pneumovirus (aMPV). The genomes of HMPV and RSV closely resemble each other, excluding a few differences in the order of the genes and the absence of the non-structural genes from the HMPV genome (Figure 1.7). HMPV is an enveloped, single stranded negative sense RNA virus that consists of 13,350 nucleotides, comprising 8 genes that code for nine proteins which include N (nucleoprotein), P (phosphoprotein), M (matrix protein), F (fusion protein), M2 (matrix protein M2-1 and M2-2), SH (small hydrophobic protein), G (glycoprotein) and L (RNA-dependent RNA polymerase) genes (van den Hoogen *et al.*, 2002).

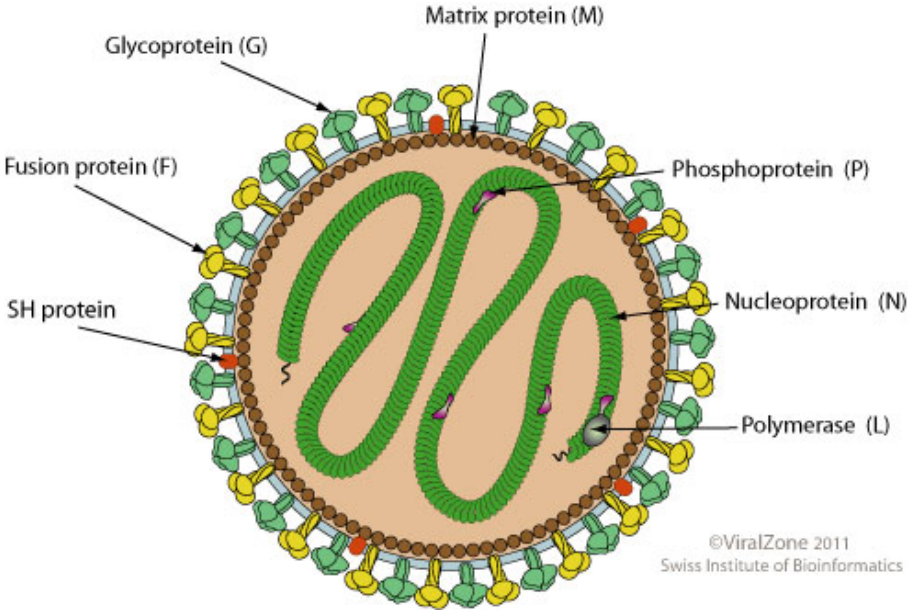


Figure 1.6: Schematic diagram of the human metapneumovirus
(Source: Van den Hoogen *et al.*, 2001)

The RNA core is surrounded by M protein and covered by a lipid envelope which contains the three surface glycoproteins (F, SH, G). The core nucleic acids are associated with the P, N, L, M2-1 and M2-2 proteins and form a nucleocapsid. With the help of G and F proteins, HMPV attaches and fuses to receptors on the cell surface. After the fusion process, the viral nucleocapsid enters into the cytoplasm of the host cell and undergoes replication. The newly synthesized viral genome assembles with the viral P, N. L, and M2 proteins, and move towards the host cell membrane. The virion then buds out of the cell, with the F, SH, and G proteins exposed on the outer side of the membrane (Chang *et al.*, 2012; Feuillet *et al.*, 2012). The P protein acts as a co-factor to stabilize the L protein, allowing the formation of the virus ribonucleoprotein (RNP) complex during virus replication. The M protein plays a crucial role in virus assembly and budding by interacting with the RNP complex. The N protein encapsidates the viral genome and protects it from nuclease activity. In addition to regulating viral transcription and replication, the M2-2

protein plays a major role in virulence by decreasing the host's innate immunity (Ren *et al.*, 2012; Schickli *et al.*, 2008).

Figure 1.7 shows the genomic maps of the *Pneumovirinae*. In HMPV, the F and M2 genes are 3' to the SH and G genes whereas in RSV, the order of these genes is reversed. The RSV genome encodes two non-structural proteins, NS-1 and NS-2-2 that are not present in the HMPV genome. The M2 genes of both viruses carry two open reading frame (ORFs) (M2-1 and M2-2). The M2 and L genes overlap in the RSV genome. The L gene of each virus, encoding the viral RNA-dependent RNA polymerase, comprises two-thirds of the viral genome and is shortened for figure clarity (Kahn, 2006).

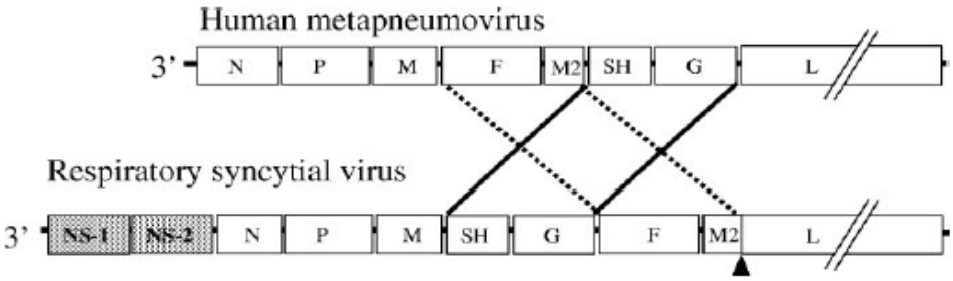


Figure 1.7: Genomic maps of the *Pneumovirinae.*
(Source: Kahn, 2006)

1.4.1.2.2 Antigenic and genetic diversity

Sequence analysis of HMPV isolates has identified two main groups, A and B; each group is further subdivided into two subgroups, A1 and A2, and B1 and B2 by phylogenetic analysis using F and G genes. Both virus groups were identified in various countries in the Americas, Europe, and Asia (Biacchesi *et al.*, 2003; Van Den Hoogen *et al.*, 2004). Subgroup A2 has been subdivided into two lineages, subgroup A2a and A2b (Huck *et al.*, 2006). It has

been suggested that these genotypes circulate in variable proportions in some areas (Gerna *et al.*, 2005; Mackay *et al.*, 2004).

The major differences between the A and B genotypes of HMPV are nucleotide polymorphisms, and the G and SH genes contain the highest concentration of these polymorphishms (Biacchesi *et al.*, 2003). Like the G gene of RSV, the G gene of HMPV displays significant strain-to-strain variability, particularly in the extracellular domain. This nucleotide variability results in significant amino acid variability, insertions that retain the reading frame, and the use of alternate transcriptional termination codons (Bastien *et al.*, 2004).

The N gene is found to be most conserved at both the nucleotide and amino acid levels (91.2% and 98.4%, respectively). The F gene was also found to be relatively conserved between subgroups (95% identity at the amino acid level between group A and B) while the G protein is highly variable with only 53% amino acid homology between group A and B (Panda *et al.*, 2014; Piyaratna *et al.*, 2011).

In an experiment done by Skiadopoulos and colleagues (2004) using rodent and nonhuman primate models, infection with a virus of one hMPV genotype protected animals against infection with a virus of the heterologous genotype. Thus, they suggested that cross-protective immunity occurs after infection with a virus of one genotype and that the F protein is a major antigenic determinant that mediates extensive cross-lineage neutralization and protection. Therefore, the two genotypes may not represent distinct serotypes and are highly related antigenically. This is analogous to the case for RSV, in which infection with a virus of either the A or B subgroup elicits an antibody response that is specific for viruses of both the homologous and heterologous subgroups (Collins *et al.*, 2001). However, in another

experiment using a ferret model, researchers reported a difference in reactivity between the two genotypes, suggesting the two genotypes were, in fact, distinct serotypes (van den Hoogen *et al.*, 2004). Thus, the antigenicity of HMPV in the natural human host remains to be determined.

It remains unclear also whether different HMPV subgroups are associated with differences in the clinical severity. Several groups have suggested that HMPV subgroup A might be associated with more severe clinical disease (Arnott *et al.*, 2013; Vicente *et al.*, 2006), while others have reported that subgroup B may cause more severe illness (Pitoiset *et al.*, 2010) and still other groups have found no evidence for differential severity caused by different HMPV lineages (Kaida *et al.*, 2006; Williams *et al.*, 2010; Xiao *et al.*, 2010). Thus the association between HMPV subgroup and disease severity is controversial. Further studies are required to better understand the clinical significance, seasonality, and molecular epidemiology of HMPV.

1.4.1.2.3 Epidemiology and seasonality

HMPV is commonly found in paediatric population, with high susceptibility rates in children less than 2 years old. About 40% of children hospitalized with HMPV infection were found to have underlying high risk conditions such as asthma and chronic lung disease (Edwards *et al.*, 2013). Although the molecular epidemiology information on HMPV has gradually accumulated, the detailed epidemiology remains unclear (Mizuta *et al.*, 2010; Pitoiset *et al.*, 2010). HMPV infections can occur throughout the year, but in several studies, it showed the epidemiological peak occurring several months later than that observed for RSV epidemics (Aberle *et al.*, 2010; Robinson *et al.*, 2005; Wilkesmann *et al.*, 2006). Outbreaks occur

mainly in the spring and winter months – January to March in the northern hemisphere and June to July in the southern hemisphere (Choi *et al.*, 2006; Pilger *et al.*, 2011). A study reported that the peak of the HMPV seasonal cases is observed between March and April following RSV and influenza infection seasons (Mizuta *et al.*, 2013). Another study reported that the HMPV infection season overlaps with that of the RSV infection season (Chan *et al.*, 2007).The seasonality of hMPV may thus vary geographically and continued surveillance over a number of years will help in understanding these patterns.

The molecular epidemiology and genetic diversity of HMPV were analysed in a study in South Africa over a 3-year period (2000-2002). Strains from both HMPV groups co-circulated in South Africa, but not all 4 subgroup viruses co-circulated in a single year. In 2000, subgroups A2 and B2 co-circulated; in 2001, A1, A2, and B2 co-circulated; and in 2002, A1 and B1 co-circulated. A switch in predominant subgroup from B2 to A1 was observed from the 2000 to the 2001 epidemic. Subgroups A2 and B2 also declined in subsequent years, and subgroup B1 emerged in 2002 (Ludewick *et al.*, 2005).

In a phylogenetic study of HMPV among children with acute respiratory infections in Kuala Lumpur, Malaysia, HMPV made up 6.5% of the 565 confirmed respiratory virus cases during the study period between 2010 and 2012 (Nor'e *et al.*, 2014). Based on the partial sequences of F fusion gene from 26 HMPV strains, 14 (54%) were subgenotype A2b, which was predominant in 2010; 11 (42%) were subgenotype B1, which was predominant in 2012; and 1 (4%) was subgenotype A2a. Subgenotype A2 was the only genotype found in a Singapore study (Loo *et al.*, 2007), while in Kolkata, India, subgenotype A2 predominated from 2006-2009 with low circulation of subgenotype B1 (Agrawal *et al.*, 2011). A2b was also dominant in southwest China in 2008-2011 (Zhang *et al.*, 2012); however, in Cambodia, subgenotype B2 predominated with low circulation of subgenotype

A2b from 2007-2009 (Arnott *et al.*, 2013). All these studies suggested that the circulation of specific subgenotypes was geographically restricted and was not similar over the Asian region and it was postulated that the alternating circulation of different HMPV subgroups is due to population immune pressure (Oliveira *et al.*, 2009).

1.4.1.2.4 Clinical presentation

The clinical manifestations of HMPV infection in young children are indistinguishable from the clinical manifestations of RSV infection. Features of HMPV infection included tachypnea, fever, cough, hypoxia, and changes on chest radiographs such as infiltrates, hyperinflation, and peribronchial cuffing (Boivin *et al.*, 2002; Robinson *et al.*, 2005; van den Hoogen *et al.*, 2003). One study reported that 50% of children with HMPV infection were diagnosed with otitis media (Williams *et al.*, 2006). Another study reported that HMPV infection was found in about 8% of children who came to the hospital with wheezing (Jartti *et al.*, 2002). Asymptomatic infection with HMPV in young children appears to be uncommon. In the study by Williams and colleagues in 2004, only 1 of 86 asymptomatic children (1.2%) tested positive for HMPV, indicating a causal role of HMPV in LRTI.

The most common diagnoses in hospitalized children who test positive for HMPV are bronchiolitis, pneumonia, and bronchitis. There are no manifestations of infection that are specific for HMPV-associated disease. In the limited number of population-based studies performed, children < 2 years old appear to be the most likely to be hospitalized due to HMPV-associated LRTI (Mullins *et al.*, 2004; Nicholson *et al.*, 2006). HMPV has been associated with severe LRTI requiring intensive care, in one case requiring extracorporeal membrane oxygenation (Ulloa-Gutierrez *et al.*, 2004), though data from some studies

suggest that, in general, the severity of disease associated with HMPV may be less than that observed with RSV (Viazov *et al.*, 2003).

1.4.1.2.5 Treatment and prevention

As with other respiratory viruses, antiviral drug therapy is difficult to implement effectively in actual practice, because viral shedding is usually already decreasing at the point in the course of infection when patients present to their medical providers. Currently, the treatments available for HMPV infection are primarily supportive. Nevertheless, ribavirin and intravenous immunoglobulin, which have activity against RSV, were tested against HMPV *in vitro* and found to have equivalent antiviral activity against these viruses (Wyde *et al.*, 2003). In addition, heparin and the sulphated sialyl lipid also have been shown to have potent anti-HMPV activity *in vitro* (Wyde *et al.*, 2004).

In terms of vaccine development, several promising vaccine candidates have been tested in animal models. A live recombinant human parainfluenza virus that contains the HMPV F gene has been shown to induce HMPV-specific antibodies and to protect experimental animals from HMPV challenge (Skiadopoulos *et al.*, 2004). A chimeric bovine/human parainfluenza virus 3 expressing the HMPV F elicits neutralizing antibodies against both parainfluenza virus and HMPV (Tang *et al.*, 2005). Recombinant HMPVs lacking the G, SH, or M2 gene or both G and SH are attenuated in cell culture, though they retain immunogenicity and elicit protective immunity in experimental animals (Biacchesi *et al.*, 2004). However, the results of these animal challenge studies should be interpreted cautiously. There are many limitations of the small-animal model for testing potential

HMPV vaccines, not the least of which is that the pathogen is highly host restricted (Kahn, 2006).

1.4.1.3 Influenza virus (IFV)

Influenza viruses are significant human respiratory pathogens that cause both seasonal, endemic infections and periodic, unpredictable pandemics. The influenza viruses are RNA viruses of the family *Orthomyxoviridae* that affect humans (Stockton *et al.*, 1998). The influenza A, B and C viruses represent three of the five genera of the family *Orthomyxoviridae* (Bouvier & Palese, 2008). Influenza A is antigenically highly variable and is responsible for most cases of epidemic influenza. Influenza A viruses are the most virulent human pathogens among the three influenza types and cause the most severe disease. The serotypes that have been confirmed in humans are shown as follow: H1N1 (Spanish flu/ Swine flu), H2N2 (Asian Flu), H3N2 (Hong Kong Flu), H5N1, H7N7, H1N2, H9N2, H7N2, H7N3, H10N7 and H7N9. Influenza B has only one species and almost exclusively infects humans. Influenza B is also characterized by antigenic changes but causes epidemics only every 5-7 years(Krejcova *et al.*, 2014). Influenza C is antigenically stable and causes only mild illness in sporadic cases among children (Matsuzaki *et al.*, 2006).

1.4.1.3.1 Virus properties

Influenza viruses are enveloped negative-strand RNA viruses with segmented genomes containing eight gene segments in influenza A and B while influenza C consist of seven gene segments of RNA, lacking a neuraminidase gene (Palese & Shaw, 2007). The virion is pleomorphic in shape with the envelope occurring in spherical or filamentous forms. The total genome is approximately 13.5 kb nucleotides in length. Unusually for a virus, the influenza virus genome is not a single piece of nucleic acid; instead, it contains seven or eight pieces of segmented negative-sense RNA which encode 11 proteins (HA, NA, NP, M1, M2, NS1, NEP, PA, PB1, PB1-F2, PB2) (Palese & Shaw, 2007). Figure 1.8 shows the influenza virus RNA genome.

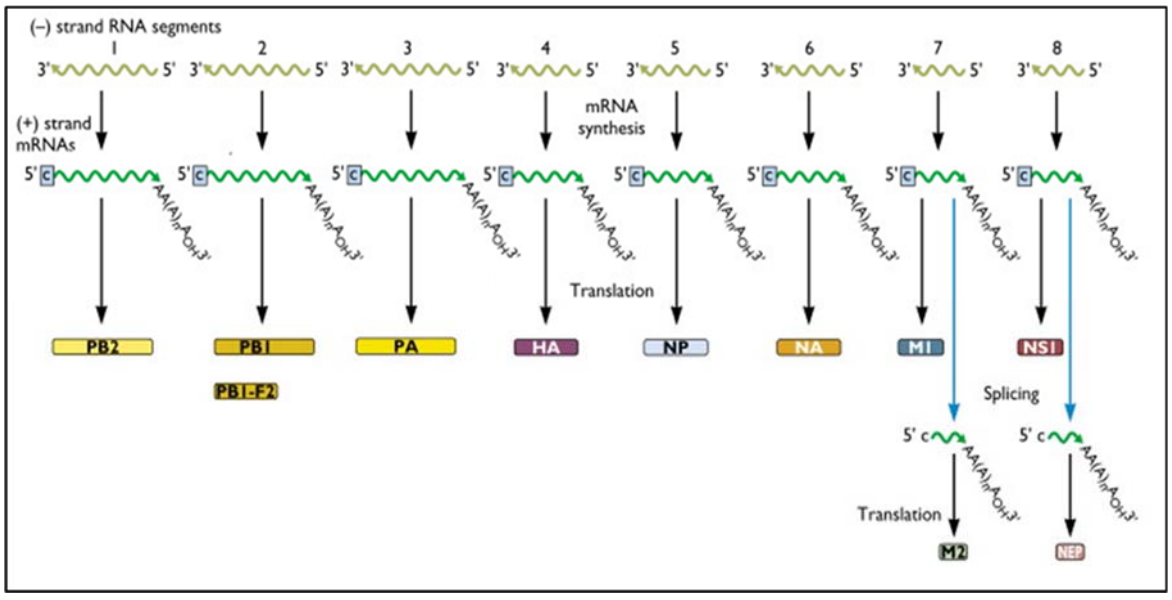


Figure 1.8: Influenza virus RNA genome
(Source: Flint *et al*., 2004)

Influenza viruses have a complex structure and possess a lipid membrane derived from the host cell. This envelope harbours the hemagglutinin (HA), the neuraminidase (NA),

and the M2 proteins that project from the surface of the virus (Figure 1.9). The matrix protein (M1) is located just beneath the envelope. The core of the virus particle is comprise of the ribonucleoprotein (RNP) complex, consisting of the viral RNA segments, the polymerase proteins (PB1, PB2 and PA), and the nucleoprotein (NP) (Palese & Shaw, 2007).

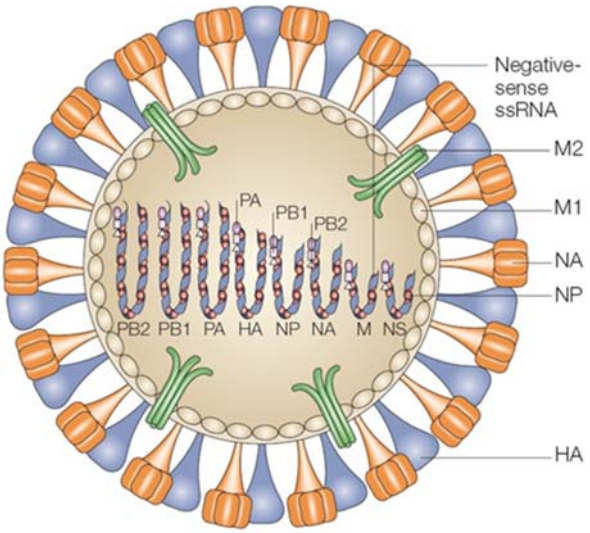


Figure 1.9: Schematic structure of influenza virus (Source: Clancy 2008).

The influenza virus hemagglutinin is a membrane-anchored glycoprotein that mediates the attachment of virus to cell-surface receptors contacting sialic acid thereby initiating viral entry and infection (Sriwilaijaroen & Suzuki, 2012). The neuraminidase is the second major glycoprotein which involved in the release of progeny virus from infected cells, by cleaving sialic acid that binds the mature viral particles (Colman, 1994). The principal antigen, hemagglutinin (HA) and neuraminidase (NA) proteins are targets for antiviral drugs. These proteins are also recognized by antibodies. The responses of antibodies to these proteins are used to classify the different serotypes of influenza A viruses. Influenza A viruses are subdivided by antigenic characterization of the HA and NA surface glycoproteins that project from the virion. Eighteen HA (H1-18) and eleven NA (N1-11)

subtypes are known. Among many subtypes of influenza A viruses, influenza A(H1N1) and A(H3N2) subtypes are currently circulating among humans (Gamblin & Skehel, 2010). Influenza B and C is not differentiated into subtypes (Biere *et al.*, 2010). Table 1.9 summarizes the influenza A virus genes and protein's function.

Table 1.9: Influenza A virus gene and proteins

RNA segment	Encoded Polypeptide (aa)	Function
1 Polymerase B2	PB2 (759)	Internal protein, virus replication, recognizes cap of host mRNAs, endonuclease (part of transcriptase complex)
2 Polymerase B1	PB1 (757) PB1-F2	Internal protein, virus replication, catalyzes nucleotide addition (part of transcriptase complex) Mitochondrial targeting and apoptosis
3 Polymerase A	PA (716)	Internal protein, virus replication, part of transcriptase complex
4 Hemagglutinin	HA (566)	Major surface glycoprotein, viral attachment, sialic acid binding, antigenic determinant, subtype specific (H1 to H16)
5 Nucleoprotein	NP (498)	Nucleocapsid protein, RNA coating, nuclear targeting, RNA transcription, interacts with RNA segments, involved in switching of mRNA to template RNA synthesis and virion RNA synthesis
6 Neuraminidase	NA (454)	Surface glycoprotein, antigenic determinant, viral release from host cells (cleaves sialic acid), subtype specific (N1 to N9)
7 Matrix	M1 (252) M2 (97)	Membrane protein stability, type (A, B, C) specific Membrane protein, viral uncoating, has ion channel activity
8 Non-structural	NS 1 (230) NS 2 (121)	NS1, non-structural protein (not detected in virions) inhibits export of poly(A)'dmRNAs and pre-mRNA splicing, IFN antagonist NS2 plays a role in export of ribonucleoproteins (RNPs) out of the nucleus, involve in regulation of virus life cycle.

(Source: Lamb & Krug, 2001)

1.4.1.3.2 Antigenic and genetic diversity

There is a hypothesis that aquatic birds are the primordial source of all influenza viruses in other species. This is supported by the phylogenetics analysis together with the findings that viruses of all known HA and NA subtypes are maintained in avian species (Webster *et al.*, 1992). The two surface antigens of influenza viruses undergo antigenic variation independently of each other. Minor antigenic changes are termed antigenic drift; major antigenic changes in HA and/or NA are called antigenic shift which is most likely to result in an epidemic (Chen & Deng, 2009).

Antigenic drift occurs as a result of point mutations in influenza viruses and refers to minor, gradual antigenic changes in the HA or NA proteins (Palese & Shaw, 2007). Accumulating over thousands of years, antigenic drift helped HA evolve into 18 separate varieties (H1 through H18) and NA into 11 (N1 through N11). A virus's subtype is a particular combination of HA and NA, for example, H1N1(CDC, 2014).

Antigenic shift is an abrupt, major change in the influenza A viruses, resulting in new hemagglutinin and/or new hemagglutinin and neuraminidase proteins in influenza viruses that infect humans. Shift results in a new influenza A subtype or a virus with a hemagglutinin or a hemagglutinin and neuraminidase combination that has emerged from an animal population that is so different from the same subtype in humans that most people do not have immunity to the new (e.g. novel) virus. Because of the segmented nature of the genome of influenza viruses, when a cell is co-infected by two different viruses of a given type, mixtures of parental gene segments may be assembled into progeny virions. This phenomenon is termed genetic reassortment (Steel & Lowen, 2014). As an example, two viruses from different species, say duck and human, infect the same host, typically a pig, and produce a

duck/human hybrid virus by exchanging RNA segments. The hybrid can gain the ability to cross species, say from pig to human, as was the case with the swine flu virus. Such a "shift" occurred in the spring of 2009, when an H1N1 virus with a new combination of genes emerged to infect people and quickly spread, causing a pandemic. When shift happens, most people have little or no protection against the new virus. While influenza viruses are changing by antigenic drift all the time, antigenic shift happens only occasionally. Type A viruses undergo both kinds of changes; influenza type B viruses change only by the more gradual process of antigenic drift. This is due to the limited host range (with no natural animal hosts other than humans and seals) of influenza B viruses as compared to influenza A viruses (Yoon *et al.*, 2014), antigenic shift does not occur in influenza B viruses and hence they have little pandemic potential (Rota *et al.*, 1990). Two antigenically distinct lineages of B viruses have established themselves in the early 1980s. The "Victoria-lineage" represented by B/Victoria/2/87 and the "Yamagata-lineage" represented by the strain B/Yamagata/16/88 (Kanegae *et al.*, 1990; Yamashita *et al.*, 1988). The two lineages co-circulated globally since the early 1980s for more than 10 years. Estimates of the rate of evolution have shown that the HA and NA of B viruses evolve more slowly than those of A viruses (Bedford *et al.*, 2015).

1.4.1.3.3 Epidemiology and seasonality

Influenza occurs globally with an annual attack rate estimated at 20%-30% in children. Every year, influenza causes millions of infections and 250,000 to 500,000 deaths (Preaud *et al.*, 2014). There are an estimated 28,000-111,500 deaths annually in children under 5

years attributable to influenza, most of which occur in developing countries (Nair *et al.*, 2011).

In the temperate zones, influenza epidemics occur annually during the winter months. In Finland, as well as in other Northern hemisphere countries, the epidemic is usually timed between December and May (Peltola *et al.*, 2003), while in the Southern hemisphere, the peak is normally between July and October (Lambert *et al.*, 2005). Typically, the epidemic lasts approximately 60 days (Neuzil *et al.*, 2000; Peltola *et al.*, 2003), although, at least during some outbreaks, influenza viruses can circulate in the community throughout the winter season (Heikkinen & Järvinen, 2003). However, in the tropics, similar seasonal distribution is not usually present (Moura, 2010). In Vietnam, for example, influenza viruses are detected year round (Nguyen *et al.*, 2009), and in Hong Kong, two separate peaks can be detected annually (Chiu *et al.*, 1999). In Thailand, influenza was associated with 10.4% of hospitalized pneumonia cases over a 4 year period, and the highest incidence was seen in children under 5 years and adult over 75 years (Simmerman *et al.*, 2009). In USA, hospitalization rates of children under 5 years with seasonal influenza were estimated at 1.4 per 100,000 (Bourgeois *et al.*, 2009), and can be compared with Southeast Asian rates in Singapore which is 0.7-0.9, Thailand (2.4), Vietnam (3.9-4.7) and Philippines (4.7) (Ang *et al.*, 2014; Le*et al.*, 2014; Simmerman *et al.*, 2009; Tallo *et al.*, 2014).

In Malaysia, influenza is identified in 2-3.2% of cases using conventional detection methods of immunofluorescence or culture (MoH, 2014; Zamberi *et al.*, 2003). However, with the use of more sensitive methods such as PCR increases influenza rates to 13% (Rahman *et al.*, 2013). Influenza is generally seen year-round in Malaysia, with no clear seasonal trends (MoH, 2014; Saha *et al.*, 2014). Influenza A is usually detected more frequently than influenza B. Seroprevalence rates of 22.3% for seasonal H1N1 and 14.7%

for seasonal H3N2 were reported in Kuala Lumpur (Sam *et al*., 2013). In a study of 132 children admitted with influenza, 16 (12.1%) had severe disease requiring intensive care or ventilation, and 3 (2.3%) died; 9 of the cases of severe disease and one death occurred in previously healthy children with no underlying chronic illness (Sam *et al.*, 2010). This suggests that seasonal influenza may cause endemic morbidity and mortality.

Four major influenza pandemics struck the world in the 20th century. By far the most devastating pandemic was the Spanish flu outbreak, which hit at the end of the First World War (Taubenberger & Morens, 2008). It spread across the globe in three consecutive waves in 1918 to 1919, killing at least 50 million people (Johnson & Mueller, 2002). The subsequent pandemics in 1957 and 1968 were milder, but nonetheless also caused a total of approximately 2 million deaths (Kilbourne, 2006).

The 1918 pandemic virus was an H1N1 strain. Its descendants circulated in humans until 1957 when they were replaced by an H2N2 subtype pandemic strain. H2N2 viruses circulated until 1968 when replaced by H3N2 viruses of the 1968 pandemic. In 1977, H1N1 strains from the pre-1957 period reappeared, and since then both influenza A subtypes H3N2 and H1N1 have co-circulated in humans (Webster *et al.*, 1992). In 1957, three genes from the circulating H1N1 human influenza virus were replaced by avian-like genes: HA, NA, and a subunit of the polymerase complex (PB1). In 1968, the HA and PB1 genes were similarly replaced (Kawaoka *et al.*, 1989; Scholtissek *et al.*, 1978).

The outbreak of Russian flu first appeared in Northern China in May 1977 and spread throughout Russia by December and the rest of the world in 1978. The virus was subsequently found to be virtually identical to one that had caused a human epidemic in 1950 (Nakajima *et al.*, 1978). Consequently, most people over 23 years old possessed antibody

to it. Thus, the pandemic was confined almost entirely to children and teenagers. The illness was quite mild and weekly attack rates at peak were about 13% in children 7-14 years old (Fox *et al.*, 1982). After the pandemic, H1N1 continued to circulate as a seasonal virus, but unlike in earlier pandemics, it failed to replace the previous seasonal strain A(H3N2). Consequently, two influenza A strains currently circulate, causing seasonal epidemics. As "Russian flu" was milder and distinct from previous pandemics in several ways, it is not always listed as a pandemic and may be considered instead as a severe seasonal epidemic.

In 1997 and 1999, new versions of avian influenza (H5N1 & H9N2) crossed into humans in China and Southeast Asia, sparking fear of new pandemics. However, these strains to date are not easily transmitted from human to human, and outbreaks have been confined to a limited number of people (Tognotti, 2009).

In 2003, a highly pathogenic avian influenza (HPAI) H7N7 virus caused a poultry epizootic in the Netherlands and spread regionally. Before the epizootic was contained, at least 86 poultry workers and three contacts had been infected and developed conjunctivitis with or without an influenza like illness (ILI). There was one fatality among those with direct contact to poultry (Fouchier *et al.*, 2004). Similarly, two people developed influenza conjunctivitis during a 2004 H7N3 HPAI outbreak in Canada (Tweed *et al.*, 2004). After re-emergence in 2003, the ongoing H5N1 HPAI epizootic continued to produce spill over infections in humans, causing concern that adaptation of this virus to humans could cause a pandemic (Taubenberger & Morens, 2008). As of December 2018, 860 confirmed cases of human H5N1 infection had been documented, of which 454 were fatal (WHO, 2018), yielding a case fatality rate of 53%.

The 2009 pandemic virus emerged in Mexico with the first clinical cases reported by CDC on April 17, 2009. The virus was soon identified as a swine origin novel reassortment A(H1N1) strain, containing genes from two different viruses circulating in pigs. Six genes were similar to those in a triple-reassortant virus isolated earlier in pigs in Northern America, and two genes were most closely related to viruses circulating in pigs in Eurasia (Dawood *et al.*, 2009). From Northern and Central America, the epidemic rapidly spread worldwide, and by the end of May, 15,510 virologically confirmed cases in 53 countries were reported to WHO (WHO, 2009). On June 11, 2009, WHO declared that the first pandemic of the 21st century had begun. The pandemic period lasted officially until August 2010.

The first pandemic case in Malaysia was reported in May 2009. Most cases occurred during an intense 6-week period in August-September, with 12,307 cases reported together with 77 deaths in 2009 (Sam, 2015). The reported cases were likely underestimated, as many cases were not confirmed in the laboratory. In an important national review of 1,362 children hospitalized with pandemic H1N1, 134 (9.8%) required intensive care, and 51 (3.7%) died. In the most affected age group of < 2 years, the hospitalization rate was 22 per 100,000 children (0.1 pre-pandemic) and the mortality rate was 1.3 per 100,000 children (Ismail *et al.*, 2011).Table 1.10 summarizes the timeline of human flu pandemics from the 20th century.

Table 1.10: Timeline of human influenza virus

Year	Name	Virus subtype	Comments
1918-1919	Spanish flu	H1N1	Major pandemic, killing more than 500,000 people in United States, and 20 million to 50 million people worldwide.
1957-1958	Asian flu	H2N2	Major pandemic, first identified in China, caused approximately 70,000deaths in United States, estimated 2 million deaths worldwide
1968-1969	Hong Kong flu	H3N2	Major pandemic, first detected in Hong Kong, estimated 1 million deaths worldwide, H3N2 viruses still circulate today.
1977-1978	Russian flu	H1N1	Reintroduce of H1N1 subtype, isolated in northern china, similar to the virus that spread before 1957.
1997	Avian flu	H5N1	First time an influenza virus was found to be transmitted directly from birds to people, with infections linked to exposure to poultry markets. 18 people in Hong Kong were hospitalized, six of whom died.
1999	Avian flu	H9N2	Caused illness in two children in Hong Kong, with poultry being the probable source.
2002		H7N2	Evidence of infection is found in one person in Virginia following a poultry outbreak.
2003		H5N1 H7N7 H7N2 H9N2	Caused two Hong Kong family members to be hospitalized, one died. First reported cases of this strain in humans, 89 people in Netherlands became ill with eye infections or flu-like symptoms. Caused a person to be hospitalized in New York Caused illness in one child in Hong Kong
2004		H5N1 H7N3 H10N7	Caused illness in 47 people in Thailand and Vietnam, 34 of whom died and becoming endemic in Asia. First reported in humans, caused illness in two poultry workers in Canada First reported in humans, caused illness in two infants in Egypt.

Table 1.10 continued

2005		H5N1	4 fatal Cambodian cases reported, 7 cases of H5N1 infection in Indonesia with 4 deaths occur. In 2005, WHO reported reported 142 cases of H5N1 infection worldwide (Thailand, Vietnam, Cambodia, Indonesia and China), with 74 deaths.
2006		H5N1	2 fatal cases reported in Turkey, one case in Iraq, 10 cases with 7 deaths in China, 7 cases and 5 deaths in Azerbaijan, four cases and two fatalities in Egypt, one case in Africa. WHO reported throughout 2006, 115 cases of H5N1 infection occur with 79 deaths.
2007		H5N1 H7N7	88 comfirmed cases occur in Indonesia, Cambodia, China, Laos, Myanmar, Pakistan and Vietnam with 59 deaths. Four cases reported in United Kingdom
2008		H5N1	40 cases confirmed in Bangladesh, Cambodia, China, Egypt, Indonesia and Vietnam
2009 2009-2012	Mexican flu (Swine flu)	H1N1 H5N1	New H1N1 strain is confirmed in Mexico. Within weeks, human infections spread to United States and cases begin occurring in other region of the world. Cases continued to be identified in Egypt, China, Indonesia and Vietnam.
2013- 2016		H7N9	A total of 808 confirmed cases including 212 deaths have been reported to WHO. All except three cases were in China, Hong Kong and Taiwan. The others involved a Chinese traveller to Malaysia and two Canadians who had visited China.
December 2013-Jan 2014	China	H10N8	On 29 January, 2014, WHO has been notified by the Chinese authorities of the detection of avian influenza A (H10N8) from a case of severe pneumonia in a 55-year-old female in Jiangxi Province. On 17 December, 2013, another avian influenza A (H10N8) has been detected in a single patient with severe pneumonia. The patient is a 73-year old female from Jiangxi Province, China. She was hospitalized on November 30 and died on December 6.

Table 1.10 continued

December 2014-2016	China	H5N6	The National Health and Family Planning Commission (NHFPC) of China has notified WHO of 15 laboratory-confirmed cases of human infection with avian influenza A(H5N6) virus.

(Source: Adapted from WHO, 2016)

1.4.1.3.4 Clinical presentation

Non-complicated influenza is typically characterized by respiratory symptoms, such as cough, rhinitis and pharyngitis, as well as systemic symptoms, including fever, weakness, malaise, headache and myalgia (Cox & Subbarao, 2000). In a prospective cohort study including 353 outpatient-treated children of ≤13 years with laboratory confirmed influenza, fever was the most common symptom. Fever at ≥38°C was reported in 90% of the children, and 20% of the children aged < 3 years had a high fever of ≥ 40°C. Rhinitis and cough was reported in 78% and 77% of the children, respectively. Other symptoms were less frequent; sore throat was reported in 36% of the children aged ≥3 years, headache was present in 26% of the children, and 7% had myalgia. 9% of the children had gastrointestinal symptoms. In that study, there were no differences in the clinical presentation between children infected with influenza A or B (Silvennoinen *et al.*, 2009).

In young children and infants, influenza may present as a sepsis-like illness (Dagan & Hall, 1984). A recent study from Finland showed that the most common reason for influenza-associated hospitalization in children < 6 months of age was a sepsis-like illness, which accounted for 52% of the admissions in this age group (Silvennoinen *et al.*, 2011). In a study among children aged < 12 years, the median duration of the illness was 5.7 days,

with a longer duration (6.7 days) seen in younger children ≤ 2 years (Whitley *et al.*, 2001). In the same study, the median duration of fever was 2.8 days.

The inoculation period in influenza ranges from 1 to 3 days. Viral shedding peaks at the onset of the symptoms or during the following 1-3 days, and then starts to gradually decline (Lau *et al.*, 2010). In children and in the immunocompromised individuals, higher viral loads have been described, and shedding may last for longer periods (Frank *et al.*, 1981; Hall *et al.*, 1979).

In children, the most common complication is acute otitis media (AOM). In different studies, AOM has been diagnosed in ~40% of children aged < 3 years, and in ~20% of children aged 3-6 years, with influenza (Heikkinen *et al.*, 2004; Neuzil *et al.*, 2002). Another frequent complication of influenza is pneumonia. Influenza-associated pneumonia may be primary viral pneumonia or a secondary bacterial co-infection (Lahti *et al.*, 2006; Ruuskanen *et al.*, 2011).

1.4.1.3.5 Treatment and prevention

Unlike many other viral respiratory infections, treatments are available for severe influenza infection. Traditional antiviral medications such as amantidine and rimantadine can be used for patients that present to hospital with severe acute symptoms, within 48 hours of onset. However, this is not usually the current treatment of choice as both amantadine and rimantadine induced significant gastrointestinal adverse effects. Neuraminidase inhibitors are preferred as they have been developed for specific treatment of influenza, however their use is limited to high risk patients such as pregnant women or those with co-morbidities (Kamali & Holodniy, 2013). Oseltamivir is an example of a commonly used neuraminidase

inhibitor that has proven effective in severe influenza infection, including novel H1N1 influenza (Govorkova *et al.*, 2011). Other examples of neuraminidase inhibitors include zanamivir, lininamivir and peramivir (Mckimm-Breschkin, 2013). One of the most effective means of reducing the population burden of influenza has been the adoption of seasonal vaccination to high risk groups in many developed countries. Vaccines utilise both inactivated and live attenuated strains of influenza and the most effective choice varied in the literature, depending on patient age and demographics (Ambrose *et al.*, 2011). Guidelines are updated regularly by the Centre for Disease Control and Prevention (CDC) in America where currently vaccination is recommended in children aged 6 months – 18 years, adults over 50 and anybody with high risk conditions (CDC, 2017).

1.4.1.4 Human parainfluenza viruses (HPIVs)

Human parainfluenza viruses (HPIVs) were first discovered in the late 1950s, when three different viruses recovered from children with lower respiratory disease proved to be unique and easily separated from influenza virus which they closely resembled. In 1959, a fourth virus was found that also met those criteria. Thus a new taxonomic group was created and called "human parainfluenza viruses". HPIVs are enveloped, negative sense, single stranded RNA viruses of the family *Paramyxoviridae.* There are two genera of HPIVs, *Respirovirus* (HPIV-1 and HPIV-3) and *Rubulavirus* (HPIV-2 and HPIV-4) (Henrickson, 2003).

1.4.1.4.1 Virus properties

HPIVs are pleomorphic enveloped viruses that are 150 to 300nm in diameter. Both genera can be separated morphologically from influenza virus by their nonsegmented thick nucleocapsids. Other genera of the *Paramyxoviridae* can be physically distinguished from HPIVs by the absence of a neuraminidase or a thinner nucleocapsid (pneumoviruses, e.g., respiratory syncytial virus (RSV) or metapneumoviruses) (Hall, 2001) (Figure 1.10).

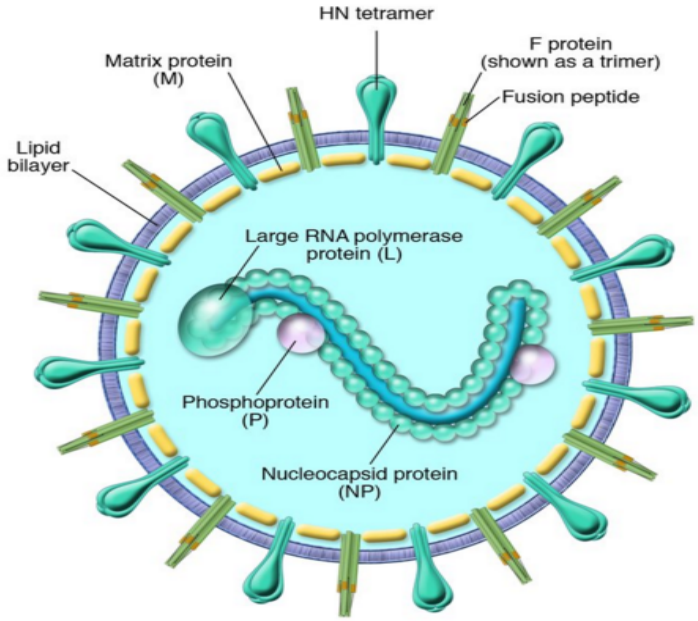


Figure 1.10: Schematic diagram of the Human Parainfluenza Virus (Source: Hall, 2001)

The HPIVs genome contains approximately 15,000 nucleotides which encode at least six structural proteins (3'-N-P/C/V-M-F-HN-L-5'). The largest HPIV protein is the "large" (L) nucleocapsid protein, followed by phosphoprotein (P). These two viral proteins and the nucleocapsid protein (N) are closely associated with the viral RNA. Two surface glycoproteins are found in all HPIVs: the hemagglutinin-neuraminidase (HN) and the fusion

protein (F). The matrix protein (M) is strongly associated with and found just beneath the viral membrane (Henrickson, 2003; Wechsler *et al.*, 1985).

N, P, and L proteins form the nucleocapsid core of HPIVs. The N binds to the viral RNA, making a template that allows the L and P proteins to transcribe and eventually replicate the HPIV genome (Chanock *et al.*, 1985). The P genes have continuous, discontinuous and overlapping cistrons and according to their coding strategy, HPIVs can be divided in two groups. In the first group, HPIV-1 and HPIV-3 encode their 500 to 600 amino acid P proteins from continuous P cistrons. The second start codon in the P gene region initiates a shorter overlapping open reading that encodes a non-structural C protein (Matsuoka *et al.*, 1991). HPIV-2, HPIV-4A, and HPIV-4B produce more than one mRNA species by editing the P mRNA which encoded the non-structural V protein (Ohgimoto *et al.*, 1990).

The surface glycoproteins (HN and F) interact with the M protein, which may direct their insertion and aggregation at specific cell membrane locations. The M protein also appears to play a role in attracting completed nucleocapsids to areas of infected cell membrane that will soon become viral envelope and may be involved in viral budding (Choppin *et al.*, 1981; Coronel *et al.*, 2001). HN functions in virus-host cell attachment via sialic acid receptors and assist virus release from cells. HPIV F protein is synthesized as a biologically inactive precursor form (F_0), which is cleaved post-translationally with cellular protease to produce an active form of two disulphide-linked subunits, F1 and F2. It is involved in virus-host cell membrane fusion which allows the viral nucleocapsid to enter and infect a host cell. This protein also needed in membrane fusion between host cells (syncytial formation) and causes hemolysis (Ortmann *et al.*, 1994). Table 1.11 summarizes the structural protein functions of HPIVs.

Table 1.11: Structural proteins of HPIVs and function

Protein	Designation	Chemical nature	Location	Function
Hemagglutinin-neuraminidase	HN	Glycosylated	Envelope	Attachment to host cell receptors, hemagglutinating and neuraminidase activities
Fusion protein	F_1F_2	Glycosylated	Envelope	Fusion, hemolysis, penetration
Nucleoprotein	NP	Phosporylated	Nucleocapsid	Tightly complexed with genomic RNA, structural component in nucleocapsid
Phosphoprotein	P	Phosporylated	Nucleocapsid	Component of polymerase complex
Large protein	L	Phosporylated	Nucleocapsid	Component of polymerse complex
Matrix protein	M	Phosporylated	Inside the envelope	Assembly

(Source: Vainionpää & Hyypiä, 1994)

1.4.1.4.2 Antigenic and genetic diversity

HPIVs are genetically and antigenically divided into type 1 to 4 in which HPIV-1 and -3 belonging to the genus *Respirovirus* and HPIV-2 and -4 to the genus *Rubulavirus*. HPIV-1, -2 and -3 are not further broken into subtypes, though different antigenic lineages and genotypes within serotypes have been described. HPIV-4 is further divided into 4A and 4B subtypes (Lau *et al.*, 2009) while HPIV-1 is divided into two major lineages (Mizuta *et al.*, 2011)and HPIV-3 is divided into three major lineages (Mizuta *et al.*, 2014). HPIV-3 included three distinct clusters (A, B and C) with subdivision of cluster C into five subclusters (C1–5) (Almajhdi, 2015). The HPIVs all induce variable levels of heterotypic antibody during infection and have common antigens. Data suggest that all four major

HPIVs serogroups (HPIV-1 to HPIV-4) have subgroups or populations that have unique antigenic and genetic characteristics. The variability and changes seen in HPIVs suggest an evolutionary pattern similar to that of influenza B virus (Henrickson, 2003).

1.4.1.4.3 Epidemiology and seasonality

HPIV are common community-acquired respiratory pathogen without ethnic, socioeconomic, gender, age or geographic boundaries. Acute respiratory infections cause 3 to 18% of all admissions to pediatric hospitals, and HPIVs can be detected in 9 to 30% of these patients (Bartolom *et al.*, 2001; Downham *et al.*, 1974; Murphy *et al.*, 1980). HPIV causes many illnesses at low rates, but patients often have severe symptoms (Rubin *et al.*, 1993). HPIVs circulates at low levels throughout the year (Billaud *et al.*, 2005). In temperate countries, an association between the HPIV types and seasonal incidence of respiratory viruses has been reported (Laurichesse *et al.*, 1999). In tropical countries, however, correlation between respiratory viral incidences and seasons are not so well defined, which may suggest that more complex interactions are involved (Olsen *et al.*, 2010)

In the United States (USA), the most common HPIV types associated with respiratory illness are HPIV-1 and HPIV-3, whereas HPIV-2 and HPIV-4 are less frequently detected. HPIV-1 infections in the US occur primarily in the autumn of odd-numbered years, while HPIV-2 infections are most commonly recognized in the autumn of even-numbered years. In contrast, HPIV-3 infections occur year-round, but peaks of illness occur each spring. HPIV-4 infections are infrequently recognized, and a clear-cut seasonality has not been established for these viruses (Fry *et al.*, 2006).

Surveys of HPIV have previously shown 3.74% and 13.2% prevalence in China and Malaysia respectively (Mao *et al.*, 2012; Khor *et al*., 2012). Previous studies found that HPIV-1 and HPIV-3 are common in China, Japan, Malaysia and Brazil (Fé *et al.*, 2008; Khor *et al.*, 2012; Mao *et al.*, 2012; Mizuta *et al.*, 2011), while HPIV-2 and HPIV-4 predominate in North America and Canada (Henrickson, 2003; Vachon *et al.*, 2006). In tropical countries, a seasonal pattern is observed whereby the proportion of HPIV-positive cases tend to be highest between January and April, but not always limited to a single type. Studies of HPIV infection in Southeast Asia also found seasonal peaks: a 2-year study in Thailand found a February-March peak, while a 4-year study in Singapore found a February-May peak (Chew *et al.*, 1998; Suwanjutha *et al.*, 1990).

1.4.1.4.4 Clinical presentation

Croup is the distinctive clinical syndrome caused primarily by HPIV-1 and HPIV-2 viruses, but it can also be seen following HPIV-3 and HPIV-4 infection. All of the HPIVs cause URIs, which include colds, otitis media and pharyngitis. URIs are the most common illnesses seen in all age groups. LRIs, including bronchiolitis and pneumonia, are more frequently seen in children and in association with HPIV-3 infection. Bronchiolitis, pneumonia and croup are the leading reasons for hospitalization, and as many as 20% of children hospitalized with acute respiratory tract infection have HPIVs infection as the cause (Weinberg, 2006).

Croup is characterized by a barking cough that usually persists for several days. Stridor and dyspnoea are also frequently seen. The incidence peaks between 1 and 2 years of age and is more frequent in boys. Approximately 10 to 25% of cases of LRI in children

younger than 5 years presents as croup. HPIV have made up between 56 and 74% of these cases, with HPIV-1 (26 to 74%) being the most frequent subtype (Denny *et al.*, 1983)).

The diagnosis of bronchiolitis is unique to pediatrics because of the size of infant's terminal airways. The predominant symptoms include fever, expiratory wheezing, tachypnea, retractions, rales and air trapping. The peak incidence of bronchiolitis is in the first year of life (81%) of cases) and at least 90% of cases are thought to be viral in origin (Murphy *et al.*, 1980). All four types can cause bronchiolitis, but HPIV-1 and HPIV-3 have been reported most commonly. In hospitalized children, HPIV-3 causes more cases than HPIV-1 (three or four times as many) and is second only to RSV as a cause of bronchiolitis and pneumonia in young infants (Henrickson, 2003). HPIV-1 and HPIV-3 each caused about 10% of outpatient pneumonias, but, similar to bronchiolitis, HPIV-3 caused a larger percentage of cases in hospitalized patients. Pneumonia can be caused by both HPIV-2 and HPIV-4, but the incidence of disease is not well described. HPIV-1 infection has been associated with secondary bacterial pneumonias in the elderly (Fiore *et al.*, 1998).

Any single HPIV can cause more than one of these LRTI syndromes to occur simultaneously or progressively in the same child. In 5 to 20% of LRTI cases, two viruses can be detected and may be associated with more severe disease (Drews *et al.*, 1997). HPIVs routinely cause otitis media, pharyngitis, conjunctivitis, and coryza. These URTI can occur singly or in any combination with LRTI. Otitis media has been shown to be associated with viral respiratory tract infections in 30 to 60% of cases. Viruses may work synergistically with bacteria to initiate otitis media or prolong symptoms (Chonmaitree *et al.*, 1990).

1.4.1.4.5 Treatment and prevention

At present, the treatment of HPIVs infections is symptomatic. Accumulating knowledge on the molecular structure and mechanisms of replication of HPIVs has accelerated research on prevention and treatment. Several strategies for vaccine development, such as the use of live attenuated, inactivated, recombinant, and subunit vaccines, have been investigated, and it may become possible to prevent HPIV infections in the near future. Nevertheless, no vaccine is currently available to protect against infection caused by any of the HPIVs (Sato & Wright, 2008).

1.4.1.5 Human rhinoviruses (HRV)

Human rhinoviruses (HRV) belong to the *Picornaviridae* family and was reclassified in the genus *Enterovirus* to correct phylogenetic inconsistencies. There were now 12 species under the *Enterovirus* genus (Table 1.12). HRV were first discovered in the 1950s and were frequently associated with the common cold. Since the development of sensitive rapid molecular techniques, the detection rate of HRV markedly improved and revealed high genetic diversity. HRV is the most common cause of upper respiratory tract infection worldwide and occur nearly year-round. However, while once thought to cause relatively benign upper respiratory tract illness, HRVs are now linked to exacerbations of chronic pulmonary disease, exacerbation of asthma, and also severe bronchiolitis in infants and children as well as fatal pneumonia in elderly and immunocompromised adults (Olsen *et al.*, 2010; Peltola & Ruuskanen, 2008).

Table 1.12: Classification, natural host ranges and associated diseases of enteroviruses

Species	Former name	Genotypes	Natural host range	Associated disease(s)
Enterovirus A	Human enterovirus A	25	Humans, monkeys	Herpangina, paralytic disease, aseptic meningitis, encephalitis, ataxia, hand, foot and mouth disease
Enterovirus B	Human enterovirus B	63	Humans, monkeys, pigs	Epidemic pleurodynia, aseptic meningitis, paralytic disease, encephalitis, myocarditis, hand-foot-mouth disease, swine vesicular disease
Enterovirus C	Human enterovirus C	23	Humans	Acute anterior poliomyelitis, respiratory disease, aseptic meningitis, encephalitis, ataxia, acute hemorrhagic conjuctivitis
Enterovirus D	Human enterovirus D	5	Humans, monkeys	Hand, foot and mouth disease, acute hemorrhagic conjunctivitis, paralytic disease
Enterovirus E	Bovine enterovirus group A	4	bovine	Reproductive, respiratory or enteric disease
Enterovirus F	Bovine enterovirus group B	6	bovine	Reproductive, respiratory or enteric disease
Enterovirus G	Porcine Enterovirus B	16	Pig, wild boar	Skin lesions
Enterovirus H	Simian enterovirus A	1		None known
Enterovirus J	Simian enterovirus	6		None known
Rhinovirus A	Human rhinovirus A	80	Human	Respiratory disease
Rhinovirus B	Human rhinovirus B	32	Human	Respiratory disease
Rhinovirus C	Human rhinovirus C	55	Human	Respiratory disease

(Source: Adapted from Knowles *et al.*, 2010; ICTV, 2013)

1.4.1.5.1 Virus properties

All picornaviruses are small (about 30nm) and spherical in shape. HRVs are composed of an icosahedral protein capsid enclosing a nonenveloped, positive sense, and single-stranded RNA of approximately 7200 bp. The genome consists of 11 genes that are translated into a single polyprotein, which is divided into three regions: P1 (containing four structural proteins VP4, VP2, VP3 and VP1), P2 (containing non-structural proteins 2A-C) and P3 (containing non-structural proteins 3A, 3B, $3C^{PRO}$ and $3D^{POL}$) (Figure 1.11). The structural proteins make up the viral capsid that encases the RNA genome, while the non-structural proteins are involved in viral genome replication and assembly to produce VPg, protease, and RNA-dependent RNA polymerase (RDRP). The VP1, VP2 and VP3 proteins responsible for the virus's antigenic diversity, while VP4 anchors the RNA core to the capsid (Jacobs *et al.*, 2013). HRVs consists of a single open reading frame joined to a 5'untranslated region (5' UTR) and a short viral priming protein (VPg) for virus replication. Type I internal ribosomal entry site (IRES) binds to the 40S ribosomal subunits to find the initiating AUG codon while 3' UTR terminate translation of the open reading frame (ORF) (Palmenburg *et al.*, 2010).

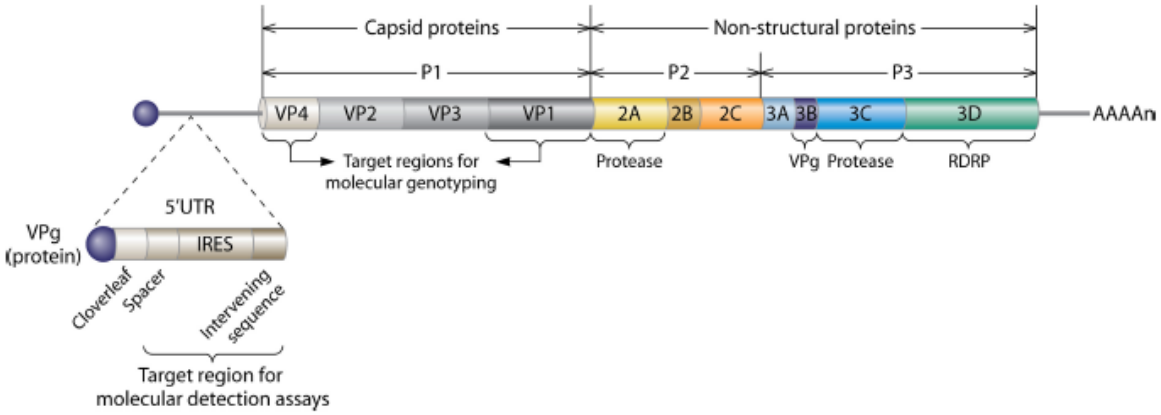


Figure 1.11: Schematic diagram of HRV genomic structure.
(Source: Adapted from Jacobs *et al.*, 2013)

HRVs were thought to be restricted to the upper respiratory tract as it predominantly replicates at 33˚C. However, it is now known that HRVs can also infect the lower respiratory tract and has been cultured from secretions in the lower respiratory tract (Halperin *et al.*, 1983). A study using in situ hybridization to localize rhinovirus infection to the lower respiratory tracts demonstrated that HRVs can penetrate and replicate in lower airway epithelium of both normal and asthmatic persons after nasal inoculation (Papadopoulos *et al.*, 2000).

HRVs are resistant to ether, chloroform, detergents and fluorocarbon due to the lack of a lipid envelope. Proteolytic enzymes, such as trypsin may destroy the infectivity of some, but not all, of the serotypes. HRVs can survive on environmental surfaces for hours to days as the infectivity of HRVs are maintained at 24˚C to 37 ˚C (Hendley *et al.*, 1988).

1.4.1.5.2 Human rhinovirus classification

Clinical specimens in the 1960s and 1970s yielded approximately 100 different HRV strains, which were subsequently serotyped and classified into two species, HRV-A and –B, based on phylogenetic sequence criteria (Palmenberg *et al.*, 2010). With the development of highly sensitive molecular methods, a much greater diversity of HRVs have been revealed than previously described and this had led to the identification of a novel species, HRV-C. HRV-C strains do not grow in standard culture cell lines which make them difficult to work with. Recently, HRV-C was successfully grown *in vitro* using sinus mucosal tissue and using a distinct cell attachment mechanism compared to HRV-A and –B (Bochkov *et al.*, 2011).

HRVs within each species have remarkable genetic and antigenic heterogeneity. By using cross-neutralization assays, species A and B isolates were originally characterized serologically into 75 and 25 serotypes respectively by 1987 (Hamparian *et al.*, 1987). However, HRV-C cannot be classified by neutralization assay because of their refractoriness to cell culture, therefore, a genetically based classification system was developed (McIntyre *et al.*, 2013).

To date, HRVs are currently organized into three species which are HRV-A, -B and –C by International Committee on Taxonomy of Viruses (ICTV). Proposals for the designation of HRV-A, -B, and -C have been published. There are now 80 HRV-A, 32 HRV-B, 55 HRV-C (C1-C55) designated (McIntyre *et al.*, 2013).

It has been shown that sequence divergence in the VP1 region correlates with serotype designation of polioviruses as well as other enteroviruses (Kilpatrick *et al.*, 1998; Oberste *et al.*, 1999). According to the study done by McIntyre *et al.* in 2013, the phylogeny

of the full VP1 region of all three HRV species reliably separated known differing types into distinct clades. This is parallel with the presence of a large number of the neutralizing immunogenic sites in HRV-A and –B that underlie their serological properties (Kistler *et al.*, 2007).

VP4/VP2 region which are well-conserved and readily amplified has been commonly used for genetic characterization of HRV in clinical samples to study its epidemiology and clinical associations (Blomqvist *et al.*, 2009; Wisdom *et al.*, 2009). The distribution of HRV types based on dataset of VP4/VP2 sequences showed similar detection frequencies in different geographical areas and this suggested that most or all HRV types circulate freely worldwide (McIntyre *et al.*, 2013).

1.4.1.5.3 Epidemiology and seasonality

HRVs have been shown to be the most frequent viruses associated with symptoms of acute respiratory illness. HRVs infections are common in all age groups and can cause respiratory illness worldwide throughout the year. Many studies have shown that HRVs infections are especially common in children (Fox *et al.*,1985; Monto *et al.*, 1987). The reported detection frequency of HRV in pediatric respiratory disease ranges from 7.7 to 37.6% (Arakawa *et al.*, 2012; Daleno *et al.*, 2013; Fry *et al.*, 2011; Huillier *et al.*, 2015). In the study done in Malaysia, HRV infection was found in 33% among hospitalized children with ALRTI (Etemadi *et al.*, 2013b).

During the 1960s, longitudinal epidemiology studies of HRVs in temperate climates reported a peak during autumn with smaller spring peak (Gwaltney *et al.*, 1966). Recent prospective studies using advanced molecular methods also replicated these findings (Miller

et al., 2007). In general, HRVs commonly cause respiratory illness during the spring, summer and autumn while influenza virus and RSV predominate in the winter. The highest incidence of rhinovirus infections has been found in September and October or alternatively in May (Fox *et al.*, 1985; Gwaltney *et al.*, 1966; Monto *et al.*, 1987). During autumn, HRVs comprise 80-90% of all common colds (Arruda *et al.*, 1997; Mäkelä *et al.*, 1998). In tropical countries, respiratory infections seem to peak simultaneously with the most intense rainfall (Monto & Johnson, 1967). All HRV species have been identified across all months, in temperate, tropical and subtropical regions (Arakawa *et al.*, 2012; Briese *et al.*, 2008).

HRV-A and HRV-C co-circulated in most months while HRV-B occurred rarely or sporadically (Onyango *et al.*, 2012). HRV-A predominates over HRV-C and HRV-B is a much less detected in most ARTI studies. HRV-B might be associated with a milder form of disease presentation and consequent of lesser rate of hospitalization (Etemadi *et al.*, 2013; Landa-Cardeña *et al.*, 2012). HRV-C has been found in cases with more severe disease and is more pathogenic since HRV-C infection could cause symptoms of greater severity than HRV-A and HRV-B infections could (Piralla *et al.*, 2009; Renwick *et al.*, 2007).

1.4.1.5.4 Clinical presentation

HRVs also known as ‘common cold virus’ which is an acute upper respiratory infection (Heikkinen & Järvinen, 2003). The first symptom is often sore throat, followed by other typical symptoms of sneezing, nasal obstruction and nasal discharge. Hoarseness, cough, fever, malaise and headache may also occur (Soile Blomqvist, 2004). The median duration of illness is seven days in young adults, but may be up to two weeks or even longer in children and in the elderly (Jartti *et al.*, 2004).

HRVs are associated with at least 70% of virus-related wheezing exacerbation, cold and flu-like illnesses. HRVs are also associated with otitis media, sinusitis and pneumonia (Arden & Mackay, 2010). HRVs is linked to otitis media in both experimental and natural settings which complicates approximately one-third of cold-like illnesses in early childhood (Winther *et al.*, 2007). In the Finnish Otitis Media Cohort study, 329 children were monitored prospectively from ages of 2 months to 2 years. HRV was detected in 41% of episodes of OM (Blomqvist *et al.*, 2002). The role of HRVs in pathogenesis of OM is still uncertain.

Strong evidence also suggests that HRVs infection is a major predisposing factor for sinusitis. 42% of adults with natural common colds had radiologically diagnosed for sinusitis on day seven of cold symptoms (Puhakka *et al.*, 1998). HRVs RNA was detected inside epithelial cells of the maxillary sinus in 50% of patients with sinus by in situ hybridization (Pitkäranta *et al.*, 2001).

Rhinovirus infections are also associated with exacerbation of chronic respiratory diseases such as asthma and chronic obstructive pulmonary disease (COPD) (Gern, 2002; Greenberg, 2002). Approximately two-thirds of respiratory virus-associated asthma exacerbations are due to HRV. In a cross-sectional study of children hospitalized with asthma exacerbations and controls hospitalized with acute respiratory illnesses, HRV was detected in 85% of cases and 33% of controls (Mak *et al.*, 2011). HRVs are the most common respiratory viral infection isolated in 24% of patients with COPD (McManus *et al.*, 2008). Another two additional prospective studies of patients hospitalized with COPD found that picornaviruses were the most common viral infection detected by RT-PCR occurring in 36 to 50% of cases (Kherad *et al.*, 2010; Rohde *et al.*, 2003).

HRVs are considered the most common viral cause after RSV among small children being hospitalized due to bronchiolitis or pneumonia (Hayden, 2004; Papadopoulos, 2004) Bronchiolitis accounts for 14% of HRV-associated LRTIs admissions to pediatric intensive care units (ICUs) (Louie *et al.*, 2009). HRV-associated bronchiolitis in infancy is an independent risk factor for recurrent wheezing at 1 year of age and development of asthma (Kotaniemi-Syrjänen *et al.*, 2003). HRVs have also been shown as a common pathogen in several clinical studies of children hospitalized with viral community-acquired pneumonia (CAP) (García-García *et al.*, 2012; Mermond *et al.*, 2012). Clinical manifestations are potentially severe particularly in children with underlying chronic medical conditions (Broberg *et al.*, 2011). In addition, HRVs was detected in 49% of children admitted to ICUs with LRTI, in which HRVs was identified as a single pathogen in almost half of the cases (Louie *et al.*, 2009).

HRV infections is also the reason for hospitalization of many of the elderly especially those with heart and lung problems (Falsey *et al.*, 2002). Besides that, HRVs are increasingly being recognized as a significant cause of acute respiratory illness in immunocompromised hosts including those with diabetes, human immunodeficiency virus (HIV) infection, malignancy, or organ transplant (Murali *et al.*, 2009).

1.4.1.5.5 Treatment and prevention

Similar with other respiratory viruses, the treatment of HRV-induced diseases is usually symptomatic and supportive. As with other viral pathogen, there are several steps in the replication cycle of viruses that are potential targets in antiviral therapy. Cell susceptibility, viral attachment, viral uncoating, viral RNA replication and protein synthesis have all been studied as targets of antiviral compounds. However, an effective vaccine evaluated in clinical trials has not yet been established. The presence of more than 100 different HRV serotypes, the lack of epidemiological data to identify the most commonly circulating HRV strains, the incomplete understanding of antigenic differences between the recently discovered HRV-C and other known serotypes besides limited animal models of HRV infection add challenges to vaccine development.

Decongestants with either antihistamine or anticholinergics appear to decrease rhinorrhea, nasal discharge, and congestion. Nonsteroidal anti-inflammatory drugs and acetaminophen may provide significant symptomatic relief. Zinc lonzenges is well-known for treating rhinovirus colds. Zinc supplementation within 24h of the onset of cold significantly reduced symptom severity and duration (Science *et al.*, 2012). Echinacea has also been a popular herbal cold remedy for decades. However, there is no conclusive evidence that Echinacea products effectively treat or prevent common cold (Karsch-Völk *et al.*, 2014). Vitamin C has also been studied for the prevention and treatment of common cold since 1942. It was demonstrated that vitamin C act as an antioxidant which may protect against the generation of oxidative stress during infections. In animal studies, vitamin C reduces the incidence and severity of bacterial or viral infections. In 2010, the Cochrane Library conducted a systemic review and meta-analysis of vitamin C for the prevention and

treatment of the common cold. They found no difference in the incidences of colds between subjects treated with vitamin C and those given placebo; however, the duration and severity of colds were reduced modestly (Hemilä & Chalker, 2013).

HRVs are transmitted through aerosol and hand contact (Dick *et al.*, 1987) Good hygiene such as proper handwashing, use of viricidal agents and avoid hand-to-mucous membrane exposure also helps decrease transmission rates. Also, avoid close contact with those who have colds or other respiratory infections (Jacobs *et al.*, 2013).

1.4.1.6 Enterovirus 68 (EV-D68)

EV-D68 is an emergent viral pathogen belonging to the species *Enterovirus D*, genus *Enterovirus*, family *Picornaviridae* as described in the previous section. EV-D68 was first described in 1962 in four Californian children hospitalized for pneumonia and bronchiolitis (Schieble *et al.*, 1967). Since then, EV-D68 had been rarely reported with a link to respiratory disease. However, since 2010, a large increase in respiratory disease associated with EV-D68 had been reported, with documented outbreaks in North America, Asia and Europe. Genetic differences were identified among circulating strains in several outbreaks, indicating the presence of multiple clades (Tokarz *et al.*, 2012).

After the initial detection, the largest and most widespread EV-D68 outbreaks were reported in August 2014 in United States. Following this, several different countries also reported an increased number of respiratory diseases due to EV-D68 (Carney *et al.*, 2015; Esposito *et al.*, 2015; Kaida *et al.*, 2011; Lang *et al.*, 2014; Midgley *et al.*, 2015).

1.4.1.6.1 Virus properties

Similar with HRVs, EV-D68 is a non-enveloped, single-stranded RNA virus of approximately 7500 nucleotides and contains a single open reading frame (ORF) encoding a polyprotein flanked by untranslated regions (UTRs) at each end; the 5ÚTR contains the highly conserved internal ribosome entry site (IRES). It encodes four structural proteins: VP1, VP2, VP3 and VP4. The VP1 gene, which encodes one of four capsid proteins, has traditionally been used to distinguish between enterovirus serotypes as it is generally considered the most variable region of the enterovirus genome. Based on molecular and biological characteristics, four human enterovirus (HEV) species are currently recognized, designated as Enterovirus A, B, C and D. Five types of EV-D isolates have been identified: EV-D68 (which is associated with respiratory disease); EV-D70 (which is associated with acute hemorrhagic conjunctivitis) (Chatterjee *et al.*, 1970); EV-D94 (which is associated with acute flaccid paralysis)(Smura *et al.*, 2007); EV-D111 and EV-D120 (which were identified in non-human primates)(Imamura & Oshitani, 2015)

EV typing is based on comparing the sequences encoding the VP1 capsid protein: viruses of different genotypes have < 75% nucleotide identity and < 85% amino acid identity (Oberste *et al.*, 1999). EV-D68 strains have been classified into three genetic groups named differently by the authors: lineages 1, 2 and 3 (Imamura & Oshitani, 2015), clades A, B, and C (Piralla *et al.*, 2014; Tokarz *et al.*, 2012), clusters 1, 2, 3 (Linsuwanon *et al.*, 2012; Meijer *et al.*, 2012), and lineage 1 which contains sub-lineages 1 and 2, and lineages 2 (Lauinger *et al.*, 2012). Numbering of the major clusters by Meijer and colleagues (2012) is synonymous to major clusters B, C and A respectively described by Tokarz *et al.* (2012).

In a study on the phylogenetic analysis of EV-D68 in Philippines, it was found that sequences belonged to lineage 2 and 3 in the phylogenetic tree based on VP1 sequences were classified into the same cluster in the phylogenetic tree for VP4/VP2 region respectively (Imamura *et al.*, 2013). Thus, analysis of phylogenetic tree based on VP4/VP2 may represent the same lineage as shown in phylogenetic tree based on VP1 region.

The 5'UTR is generally the most conserved region in enterovirus and thus is used as a target region for RT-PCR and sequencing for screening purposes (CDC, 2011). The 5'UTR sequences of EV-D68 is similar to those of HRV; therefor some of the EV-D68 strains were detected by PCR and sequencing using primers designed against the 5'UTR of HRV. It was reported that the 5'UTR sequences were similar among the strains detected during the same outbreak (Imamura *et al.*, 2011; Renois *et al.*, 2013). However, some genetic variations have been reported at the 3' end of the 5'UTR, the spacer region between the internal ribosome entry sites and the open reading frame of the VP4 region. Some strains were reported to have a 24-nucleotide deletion at positions 681-704 of the Fermon strain (Figure 1. 12b) and some of them had an additional 11-nucleotide deletion at positions 721-731 (Figure 1.12c) (Kaida *et al.*, 2011; Tokarz *et al.*, 2012). Strains of lineage 1 were likely to have deletions at both positions 681-704 and 721-731. In contrast, lineage 3 strains had deletions only at positions 624-704, whereas those of lineage 2 had no deletions in the 5'UTR . Besides, that, lineage 3 share a unique sequence characteristic in which there is nucleotide deletions at positions 2806-2808 in the VP1 region compared to the Fermon strain which results in an amino acid deletion at that position (Figure 1.12b). It is believed that these deletions might affect the virulence of the virus by enhancing translational efficiency which might be related with the recent increase in EV-D68 cases worldwide (Tokarz *et al.*, 2012).

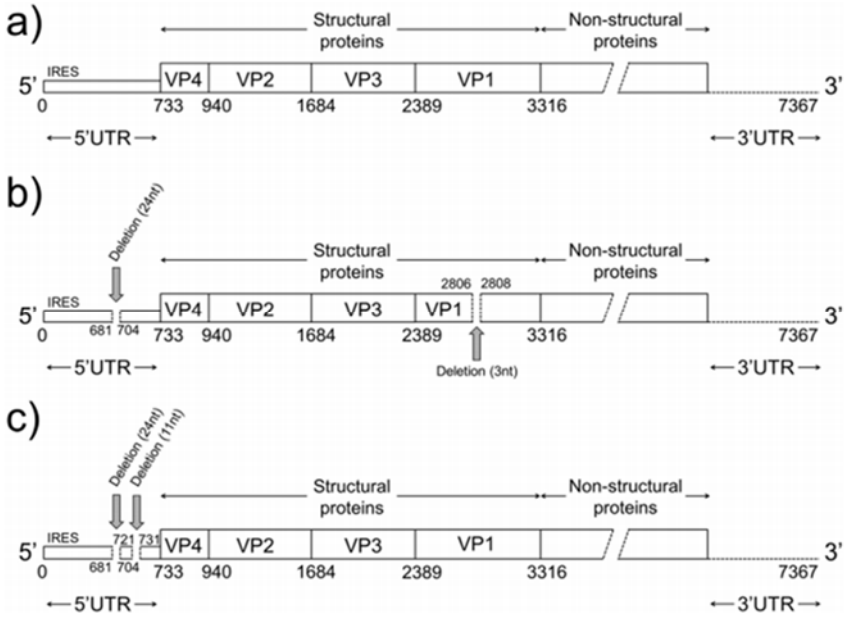


Figure 1.12: Genome structure of EV-D68. The genome structures of (a) the Fermon strain and lineage 2, (b) lineage 3, (c) lineage 1 of EV-D68. Each genome region is indicated with a bar, and the nucleotide positions of these regions are annotated with numbers below the bars.

(Source: adapted from Imamura & Oshitani, 2015)

EV-D68 is considered an atypical EV, sharing some biological characteristics with HRVs. EV-D68 grows at lower temperatures at 33˚C and does not tolerate acidic conditions unlike the majority of EVs. EV-D68 is biologically more similar to HRVs. Thus, EV-D68 was initially named HRV-87 until genetic and antigenic analysis led to its re-classification as an enterovirus (Oermann *et al.*, 2015).

1.4.1.6.2 Epidemiology

After EV-D68 initial identification in 1962, only 26 clinical isolates of EV 68 were reported in USA between 1970 to 2005 (Khetsuriani *et al.* 2006). However, over the past 7 years, outbreaks in Japan, the Philippines, and the Netherlands as well as epidemic clusters in the United Kingdom have implicated EV-D68 as an emerging respiratory pathogen (Ikeda *et al.*,

2012; Imamura *et al.*, 2011; Jacobson *et al.*, 2012; Kaida *et al.*, 2011; Meijer *et al.*, 2012; Rahamat-Langendoen *et al.*, 2011).

In late 2008 and early 2009, EV-D68 was detected in 21 patients in Philippines, two of whom died (Imamura *et al.*, 2011). In Japan, 14 cases were reported in the autumn of 2010 (Kaida *et al.*, 2011). In the USA, EV-D68 clusters were reported in several states in 2010 (CDC, 2011; Tokarz *et al.*, 2012). In the Netherlands, 24 EV-D68 cases were recorded between August and November 2010 (Rahamat-Langendoen *et al.*, 2011). Figure 2.13 showed the occurrence of EV-D68 in different countries between 2008 and 2010.

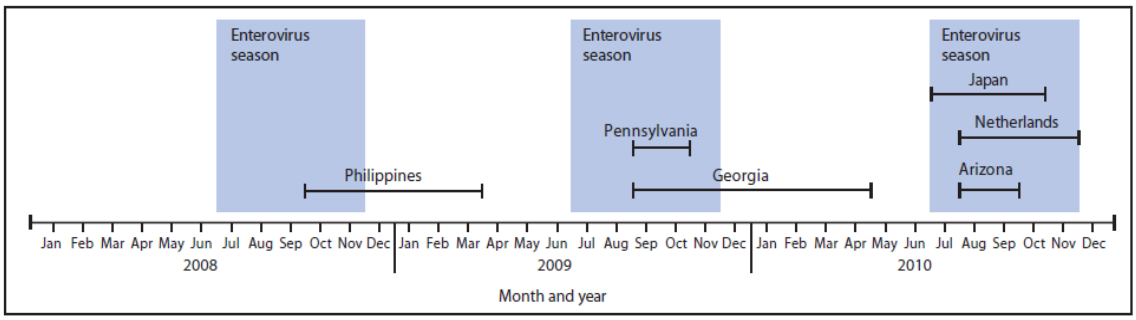


Figure 1.13: Occurrence of EV-D68 by month, duration and geographic location-Asia, Europe, and United States, 2008-2010.
(Source: Adapted from CDC, 2011).

In the Philippines, EV-D68 was identified three times: first in 2008-2009, second in 2011 and third in 2013-2014. It is possible that EV-D68 outbreaks occur in cyclic patterns at 2-year interval in Philippines (Imamura & Oshitani, 2015). Increased detection of EV-D68 have recently been reported in USA since mid-August 2014 (Brown *et al.*, 2014).

Based on literature review, according to phylogenetic analysis of VP1 region, strains belonging to lineages 1 and 2 are likely to form clusters containing viruses from the same geographical origins. For example, strains from Yamagata, Japan form a cluster in lineage 2, and those from Italy form a cluster in lineages 1 and 2. In contrast, strains belonging to

lineage 3 are less likely to show geographical clustering patterns. These suggest that transmission at the global level might be more common for lineage 3 than for lineages 1 and 2 (Imamura & Oshitani, 2015).

In Yamagata, Japan, strains belonging to all three lineages were detected in 2010. In Italy, England, Thailand, and the Philippines, co-circulation of lineages 2 and 3 strains were reported. Strains of only one lineage were detected each year in some study sites including New Zealand (lineage 3 only), New York, USA (lineage 3); China (lineage 3); and Osaka, Japan (lineage 1). In the majority of EV-D68 cases reported worldwide, children represent the main population affected because these were the patients who are commonly recruited into a study.

1.4.1.6.3 Clinical presentation

EV-D68 has been reported to cause mild to severe upper and lower respiratory illnesses. The infection is characterized by sudden onset of the disease, and the major respiratory symptoms include fever, rhinorrhoea, sneezing, malaise, cough and sore throat (Foster *et al.*, 2015; Piralla *et al.*, 2014); pneumonia and asthma exacerbation represent the main complications due to EV-D68 (Torres *et al.*, 2015; Reiche *et al.*, 2015). In a national EV-D68 surveillance study conducted in The Netherlands from 2011 to 2014, EV-D68 was detected in 0.57% in patients with respiratory samples and 0.17% of the healthy individuals was positive for EV-D68 too. Higher detection rate of EV-D68 among patients than healthy controls suggest that EV-D68 is more likely to be associated with respiratory diseases in humans. Besides that, EV-D68 positive acute respiratory infections were commonly accompanied with severe respiratory symptoms such as dyspnea and bronchitis (Poelman *et al.*, 2015).

In Philippines, the EV-D68 detection rate was higher among patients hospitalized with pneumonia than among outpatients with other respiratory infections (Kaida *et al.*, 2011). During the recent outbreaks in Kansas City and Chicago in 2014, many of the hospitalized children had clinical manifestations of severe illness, including difficulty in breathing, hypoxaemia and respiratory distress, largely without fever. Most of the children were admitted to the intensive care unit and required mechanical ventilation (Midgley *et al.*, 2015).

The overall data from different countries suggest that EV-D68 infections are more likely to be associated with severe and life-threatening respiratory diseases than that caused by other EVs. Many EV-D68 infections appear to be more severe in patients with underlying conditions such as prematurity or chronic diseases, in immunocompromised children and in those with a history of asthma or wheezing (Meijer *et al.*, 2012; Midgley *et al.*, 2015; Rahamat-Langendoen *et al.*, 2011).

EV-D68 has been associated with central nervous system diseases in some reports. Many cases of neurological complications associated with respiratory illnesses in children have been reported in the United States and Canada (Ayscue *et al.*, 2014; Midgley *et al.*, 2015). In Colorado, acute flaccid paralysis and cranial nerve dysfunction in children associated with an outbreak of EV-D68 was reported by Messacar *et al.* (2015). EV-D68 has been detected in the cerebrospinal fluid (CSF) in a case of a 5-year old boy who died due to meningoencephalitis and pneumonia (Kreuter *et al.*, 2011). In Norway, France, and Europe, cases of severe neurological complications linked with EV-D68 have been reported as well (Lang *et al.*, 2014; Pfeiffer *et al.*, 2015).

1.4.1.6.4 Treatment and prevention

No specific antiviral therapy is available to treat EV-D68 infection. In fact, treatment for EV-D68 infections is mainly supportive. Mild cases of EV-D68 infections are self-limited and require no treatment other than supportive therapies, whereas children with more severe illness require hospitalization and often intensive care interventions, including mechanical ventilation (Murray *et al.*, 2015).

The Centres for Disease Control and Prevention (CDC) in the United States has tested many antiviral drugs that appeared to have activity against some EVs (Oermann *et al.*, 2015). Among the 16 tested drugs, only four (KR-22865, rupintrivir, V-7404 and DAS181) were found to have strong inhibitory activity. However, rupintrivir and KR-22865 were abandoned and are not currently being developed. V-7404 is being developed in combination with pocapavir for the treatment of poliovirus infection and DAS 181 is being developed to treat influenza and parainfluenza infections. The last two compounds are possible drug candidates for alleviating EV-D68 outbreaks (Rhoden *et al.*, 2015). As there is no vaccines available for EV-D68, conventional infection control measures in both community and healthcare settings are recommended.

1.4.1.7 Human coronaviruses (HCoV)

Human coronaviruses are enveloped, positive single stranded RNA viruses of the family *Coronaviridae*. The family *Coronaviridae* has been categorized into sub-family *Coronavirinae* and *Torovirinae.* HCoV mainly causes respiratory or enteric disease in mammals and birds in a species-specific manner. This family of viruses is responsible for a variety of severe diseases in domesticated animals. In recent years, evolution of HCoVs due to factors such as urbanization and poultry farming have permitted the frequent mixing of different species which potentially could lead to crossing of the species barrier and results in genomic recombination of these viruses that could circulate globally within human population (Jones 2013). HCoVs have been reported causing life-threatening pneumonia and bronchiolitis especially in children, elderly and immunocompromised patients (Pene *et al.*, 2003, Walsh *et al.* 2013, Gorse *et al.*, 2009). Besides respiratory illnesses, they may also cause enteric and neurological diseases (Arbour *et al.*, 2000; Smuts, 2008; Graham *et al.*, 2013).

1.4.1.7.1 Virus properties

Coronaviruses have the largest known viral RNA genomes, with a length of 27 to 32kb (Masters, 2006). The single-stranded genome is capped and polyadenylated. This enveloped virus carries extended spike proteins on the membrane surface, providing the typical crown-like structure (crown= corona) seen by electron microscopy (Pyrc *et al.*, 2004). Figure 1.14 is a schematic diagram showing morphological features of coronavirus particles and its envelope glycoproteins. The coronaviruses share a conserved organization of their RNA

genomes. The 5'two-thirds of the genome contain the large 1a and 1b open reading frames (ORFs) encoding the proteins necessary for RNA replication (the non-structural proteins), whereas the 3'one-third contains the ORFs coding for the structural proteins: spike protein (S), envelope protein (E), membrane protein (M) and nucleocapsid protein (N). Some members of betacoronavirus subgroup (HCoV-OC43 and HCoV-HKU1) also code for a shorter spike-like protein called hemagglutinin esterase (HE) (Figure 1.14) (Pyrc *et al.*, 2004; Siddell, 1983).

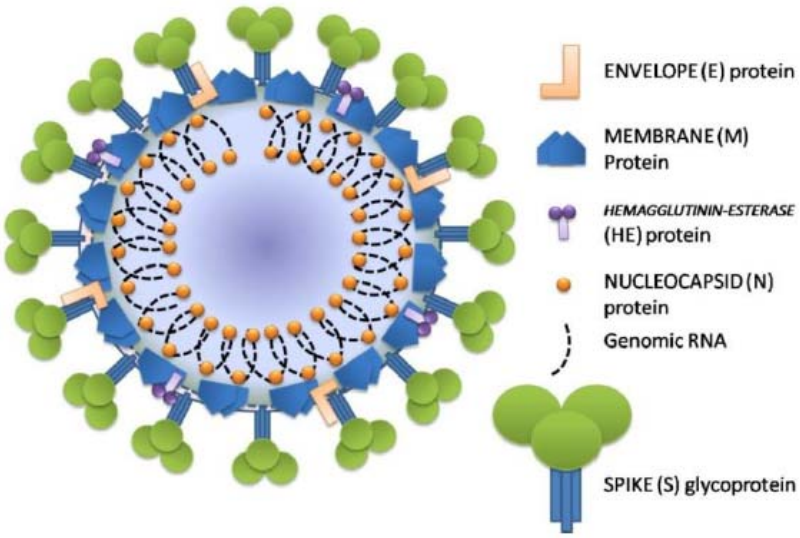


Figure 1.14: Schematic diagram of Coronavirus virions
(Source: Masters, 2006)

The RNA genome starts at the 5' end with a leader RNA (L, 60-80 nt) followed by 5'(200-600 nt) UTR and then by two overlapping ORF, 1a and 1b, which encompass 60% of the genome. These encode for polyproteins *rep 1a* and *rep1b* by means of ribosomal frameshifting (Holmes & Lai, 1996), which can be processed proteolytically to non-structural protein, viral RNA dependent RNA polymerase (RdRp), helicase, papain like proteases ($PL1^{pro}$ and $PL2^{pro}$), and main protein (M^{Pro}) to name a few. The non-structural genes are followed by CoV structural genes invariantly in the order 5'-SEMN-3' (Figure

1.15). At least eight accessory genes are intercalated among the structural genes, which may be essential for natural infection though not *in vitro*. Finally a 3' UTR (200-500 nt) is flanked by a polyadenylation signal. The UTRs share genome replication and encapsidation signals with adjacent and internal coding regions (de Groot *et al.*, 2011).

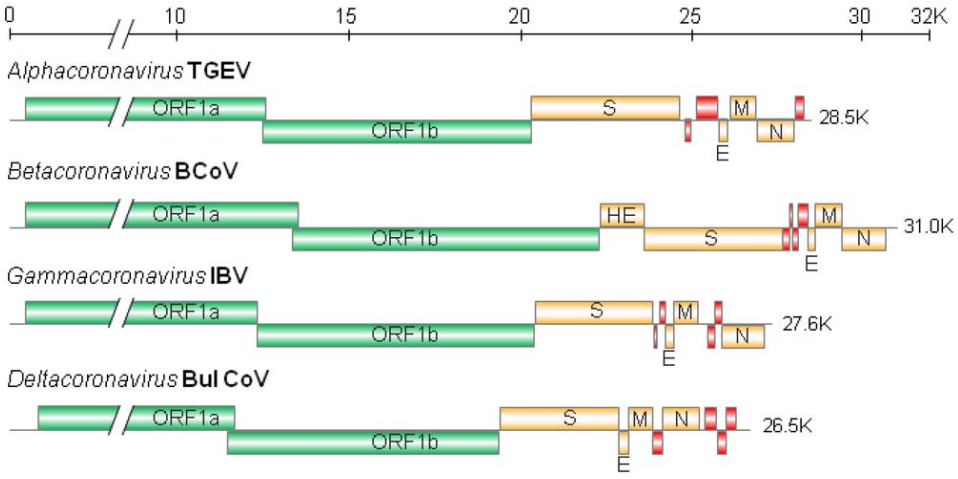


Figure 1.15: Genome organizations of various Coronavirus genera. The first two-thirds of the genome contains open reading frame (ORF) 1a and ORF1b, which encode the replicase/transcriptase proteins (Green). The remaining one-third of the genome encodes four structural proteins: spike (S), envelope (E), membrane (M), and nucleocapsid (N) proteins (Orange). Some *betacoronaviruses* have an additional hemagglutinin-esterase (HE) gene (orange). The genome of each genus or species has a set of unique accessory proteins (red).

(Source: Ujike & Taguchi, 2015)

The spike (S) is a type I membrane protein which anchors the virus particle to host cell membrane receptors, induce membrane fusion and is also a major target of CoV neutralizing antibodies (Masters, 2006). The membrane protein (M) is a type III glycoprotein which helps in virus assembly, packaging genomic RNA into nucleocapsid (Lai *et al.*, 2006). Envelope protein (E) is an integral membrane protein that forms pentamers and has cation-selective ion channel activity (Wilson *et al.*, 2004). It is required in virion assembly and morphogenesis and is a virulence factor in SARS-CoV (Masters, 2006, DeDiego *et al.*, 2007). Nucleocapsid Protein (N), is a RNA binding phosphoprotein which

is responsible for genome encapsidation, RNA synthesis, translation, and exhibits RNA chaperone activity besides acting as type I interferon antagonist (Lai *et al.*, 2006). The Hemagglutinin-esterase protein (HE) is a short spike-like glycoprotein found only in lineage A of the *Beta*-CoV genus. It shares 30% amino acid sequence similarity with that of influenza C virus. It may help initial adsorption of the CoV to the host cell membrane, but it cannot initiate infection in the absence of spike protein (Lai *et al.*, 2006).

1.4.1.7.2 Human coronavirus classification

The recent viral taxonomic convention established CoV species on the basis of genetic diversity separates these viruses into three genera (de Groot *et al.*, 2011), *Alpha*-, *Beta*-and *Gamma*-CoV with a tentative new member, *Delta*-CoV (Fig 1.16). At present, there are six known human coronaviruses: HCoV-229E, HCoV-OC43, severe acute respiratory syndrome (SARS)-CoV, HCoV-NL63, HCoV-HKU1 and MERS-CoV. The human coronaviruses (HCoVs) are in two of these genera: *Alpha*-CoV (formerly known as Coronavirus group 1 including subgroups 1a and 1b), which are prototyped by HCoV-229E and HCoV-NL63; *Beta*-CoV (previously known as coronavirus group 2) which include HCoV-HKU1, HCoV-OC43, MERS-CoV and SARS-CoV (de Grooot *et al.*, 2011).

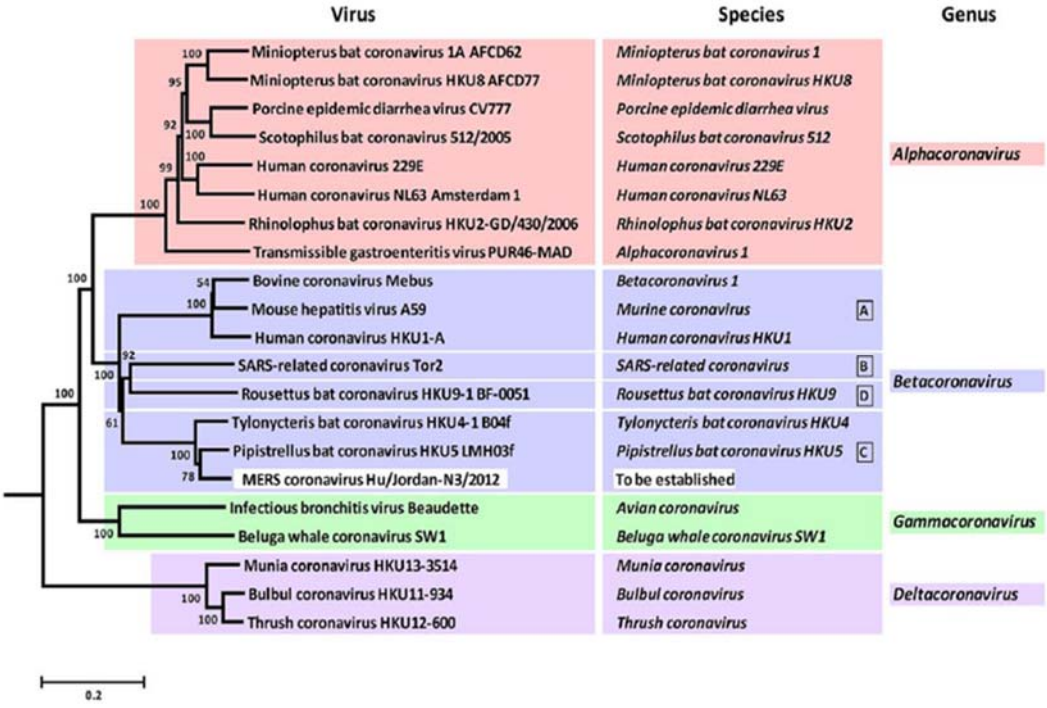


Figure 1.16: Phylogenetic relationships among CoV. A rooted neighbour-joining tree generated from amino acid alignments of *Coronaviridae*-wide conserved domains in replicase polyprotein 1ab of 21 CoV. Each of these is representative of a currently recognized CoV species; Equine torovirus Berne served as the out-group. Virus names are given with strain specifications; species and genus names are in italics as per convention. The tree shows the four main colour coded monophyletic clusters, respective to genera Alpha-, Beta-, Gamma-, and Delta-CoV. Also indicated are Beta-CoV lineages A through D (corresponding to former CoV subgroups 2A through D). Bootstrap values (1,000 replicates) are indicated at branch points. The tree is drawn to scale (scale bar, 0.2 amino acid substitutions per site).

(Sources: Adapted from de Groot *et al.*, 2011, de Groot *et al.*, 2013)

HCoV-229E and HCoV-OC43 were first described in the mid-1960s and, for over 40 years, they were believed to be the only representatives of human coronaviruses (Hamre & Procknow, 1966; Tyrrell & Bynoe, 1965). The SARS epidemic began in 2003 in the Guandong province of China. The SARS-CoV probably originated from a wild animal reservoir, likely bats, and was transmitted in a zoonotic event to humans (Lau *et al.*, 2005). HCoV-NL63 and HCoV-HKU1 were identified in 2004 and 2005 respectively. Both viruses have spread worldwide and display a spectrum of disease similar to those described for

HCoV-229E and HCoV-OC43 (van der Hoek *et al.*, 2004; Woo *et al.*, 2005). Recently, another novel coronavirus associated with severe acute respiratory syndrome has emerged in the Middle East, this novel coronavirus now named as Middle East Respiratory Syndrome Coronavirus (MERS-CoV) (Zaki *et al.*, 2012). The currently circulating HCoV can probably all be classified as common cold viruses but a more severe lower respiratory tract infection is frequently observed in young children, patients with underlying disease and the elderly (Chiu *et al.*, 2005; Gerna *et al.*, 2006).

1.4.1.7.3 Epidemiology and seasonality

Human coronaviruses are increasingly recognized as respiratory pathogens associated with a broadening range of clinical outcomes. HCoVs circulate worldwide and the prevalence of the different species varies by year and geographical region (Greenberg, 2011). Recent reports show that HCoVs have been detected in Hong Kong (Lau *et al.*, 2006), Italy (Gerna *et al.*, 2007), France (Vabret *et al.*, 2008), USA (Dominguez *et al.*, 2009), UK (Gaunt *et al.*, 2010), Netherlands (Esposito *et al.*, 2006), Finland (Risku *et al.*, 2010), and China (Ren *et al.*, 2011) from young children and adults. In the study done by Gaunt *et al.*, (2010), three coronaviruses were more frequently detected as the sole pathogen in subjects with LRTI than in those with no respiratory symptoms and were detected as often as HPIV-1 and HPIV-2. HCoV-OC43 was the most commonly detected coronavirus followed by HCoV-NL63 and HCoV-HKU1, with similar moderate detection frequencies, and HCoV-229E, with a comparatively low detection frequency (Lau *et al.*, 2006; Gaunt *et al.*, 2010; Prill *et al.*, 2012). However, in some regions for example, in the Rocky Mountain region, the

predominant strains were NL63, OC43 and 229E, while in the Pacific Northwest, HKU1 was predominant (Dominguez *et al.*, 2009; Kuypers *et al.*,2007).

Prevalence of HCoV-229E, HCoV-NL63 and HCoV-OC43 was 0%, 5% and 5% respectively in children less than 2 years of age with LRTI and 0%, 1.4% and 2.8%, respectively in children with URTI in New York (Talbot *et al.*, 2009). That study suggested that HCoV-OC43 and HCoV-NL63 may be associated with LRTI in children. In support of the association another similar study indicated that the later viruses occur in high frequencies in children with LRTIs and URTIs and that these viruses are prone to cause severe illness in children (Lee & Storch, 2014).

Human coronavirus infections occur mainly in winter, with a short incubation time (Gerna *et al.*, 2007). HCoV-NL63 detection peaked in winter and spring in France & Canada (January to April), which contrasts with the summer-to-autumn seasonality reported to occur in the tropics as in Hong Kong (Vabret *et al.*, 2008; Bastien *et al.*, 2005; Chiu *et al.*, 2005; Lau *et al.*, 2006). Prill *et al.* (2012) reported that in US, among children < 5 years of age hospitalized for ARI/fever, OC43 tended to circulate from fall to winter, while 229E was not detected in the first four years of the study and only one cluster of HKU1 during a single winter. In a study by Kuypers *et al.*, (2007), HKU1 was more likely to occur from October to February and 229E from December to June in United States. In a study of both upper and lower respiratory tract illnesses in children in Colorado by Dominguez *et al.*, (2009), HKU1 was more likely to occur from February to May and 229E from February to June.

1.4.1.7.4 Clinical presentation

Infections with coronaviruses have been documented in various studies from both children and adults, including symptomatic and non-symptomatic individuals. HCoV-NL63 was frequently associated with croup together with influenza virus or HPIV-1, in children less than 5 years of age with acute respiratory disease (Choi *et al,* 2006). According to Chiu *et al.*, (2005), HCoV-NL63 infection can present as croup, asthma exacerbation and febrile seizures. The peak age for hospitalization with HCoV-NL63 infection was 2-3 years, which is similar to that for HCoV-OC43 infection. In another study done in Korea between 2004-2006 among children hospitalized with acute respiratory disease, the prevalence of HCoV-NL63 in children under 16 years of age was 1.7%, of which 31% of the cases presented with croup and 21.4% had bronchiolitis (Han et al, 2007). A prospective study in South Africa conducted among consecutive children presenting with acute wheezing to a pediatric hospital from May 2004 to November 2005 found that the prevalence of HCoV-NL63 was 2.4% of positive viral detections (Smuts et al 2008).

HCoV-OC43 was also associated with exacerbation of asthma and pneumonia, as well as upper respiratory tract disease. 11% of children with HCoV-OC43 infections were found to have asthma exacerbation compared to 14% of children with HCoV-NL63 infections (Chiu *et al.*, 2005). Jean *et al.*, (2013) revealed that HCoV-OC43 infections generally caused upper respiratory tract infection, but can be associated with lower respiratory tract infection especially in those co-infected with other respiratory viruses among pediatric population.

HCoV-HKU1 cases were first described among elderly patients with major underlying diseases, in particular of the respiratory and cardiovascular systems (Woo *et al.*,

2005). The prevalence of HCoV-HKU1 was 19.2% in patients with acute respiratory disease (Gerna et al 2007). Although a high prevalence of HCoV-HKU1 was reported, some studies have indicated lower detection rates of HCoV-HKU1 (Woo et al 2005). Studies suggested that HCoV-HKU1 infection may aggravate the conditions of persons with an underlying disease, thus requiring hospitalization (Esper *et al.*, 2006; Vabret *et al.*, 2006). HCoV-HKU1 infection usually present with symptoms such as rhinorrhea, fever, coughing, wheezing and disease manifestations include bronchiolitis and pneumonia (Lau *et al.*, 2006; Sloots *et al.*, 2006). Vabret *et al.* (2006) also suggested that HCoV-HKU1 might also be involved in gastrointestinal disease. A study of children with acute respiratory tract infections demonstrated an interesting association between HCoV-HKU1 infection and febrile seizure (Lau *et al.*, 2006). These symptoms were more frequently observed in HCoV-HKU1 infected children than HCoV-OC43 infected children. HCoV-229E differed clinically from other coronaviruses and other respiratory viruses in general whereby 70% of detections were in samples from immunocompromised patients (Gerna *et al.*, 2006; Pene *et al.*, 2003).

It is well known that all HCoVs cause respiratory infections. SARS-CoV is the most aggressive, although the disease seems to be substantially less severe in children than in adults. In patients <12 years of age, the clinical course of SARS was generally milder and shorter than in those ≥12 years: no death was reported, only 5% of the infected children were admitted to an intensive care unit, and <1% required mechanical ventilation (Dowell & Ho, 2004). HCoVs were believed to cause only mild upper respiratory tract infections (URTIs) in children and that only in premature infants and children with a chronic underlying disease could severe LRTIs develop (Monto & Lim, 1974). Gerna *et al.* (2006) found a high incidence of HCoV-229E, HCoV-OC43, and HCoV-NL63 infections in infants and children

with bronchitis, bronchiolitis, or pneumonia, but most of the LRTIs were demonstrated in children co-infected with HCoVs and other respiratory viruses.

In 2012, a novel etiological agent named Middle East Respiratory syndrome coronavirus (MERS-CoV) was identified (de Groot *et al.*, 2013). MERS-CoV is one of six known human coronaviruses that cause respiratory disease in humans and, with a mortality rate > 35% (WHO, 2016). MERS-CoV is linked with more severe disease in older people, people with weakened immune systems, and those with chronic co-morbidities such as cancer, chronic lung disease and diabetes. Interestingly, MERS-CoV cases have been reported mainly in adults, with children rarely affected (Chafekar & Fielding, 2018).

1.4.1.7.5 Treatment and prevention

There is currently no treatment or vaccine available to fight HCoVs infections. Various approaches were used during SARS-CoV, but none was really successful and targeted (Geller *et al.*, 2012). Treatment was essentially based on supportive care. Chloroquine has potent antiviral activity against the SARS-CoV which has been shown to have similar activity against HCoV-229E and HCoV-OC43 in cultured cells (Kono *et al.*, 2008; Keyaerts *et al.*, 2009). However, efficacies in humans are yet to be studied.

Preventive measures are the same as for most other viral infections, which include hygiene handwashing and the careful disposal of materials infected with nasal secretions. Several antiseptic or disinfectant solutions commonly used in hospitals and households for example chloroxylenol, benzaalkonium chloride, and cetrimide have been shown to be ineffective against coronaviruses (Wood & Payne, 1998).

1.4.1.8 Human adenovirus (HAdV)

Human adenovirus was first isolated in 1953 from adenoid tissue-derived cell cultures, hence naming it as adenovirus (Rowe, 1953). Human adenovirus belongs to the genus *Mastadenovirus* of the *Adenoviridae* family (Norrby *et al.*, 1976). Adenoviruses have traditionally been associated with respiratory tract infections, eye infections and diarrhea. Recently, they have been associated with obesity and heart infections. Adenoviruses commonly cause respiratory and gastrointestinal illness among children and young adults. It also has been increasingly recognized as significant viral pathogens especially in those immunocompromised individuals. More than 50 serotypes of HAdV have been identified. Different serotypes correlate with different clinical manifestations of infection (Lynch *et al.*, 2011).

1.4.1.8.1 Virus properties

Adenoviruses are non-enveloped, linear double-stranded DNA genome of approximately 36 kb with an icosahedral structure about 90nm in diameter. The outer structure of the virus is comprised of 240 hexons and 12 pentons at the vertices of the icosahedron. Adenovirus fibers of species-specific lengths extend from the penton and are associated with hemagglutination properties (Berk, 2007).

The eleven structural proteins are designated II to XII and the major soluble antigens characteristic of adenovirus infections are named hexon (polypeptide II), penton base (polypeptide III) and fiber (polypeptide IV) which are the major proteins of the viral coat. Polypeptide IIIa, VI, VIII and XI are the minor coat proteins. The other four polypeptides

which are polypeptide V, polypeptide VII, μ and the terminal protein TP, are packaged with the genomic DNA in the viral core (Figure 1.17) (Rux & Burnett, 2004).

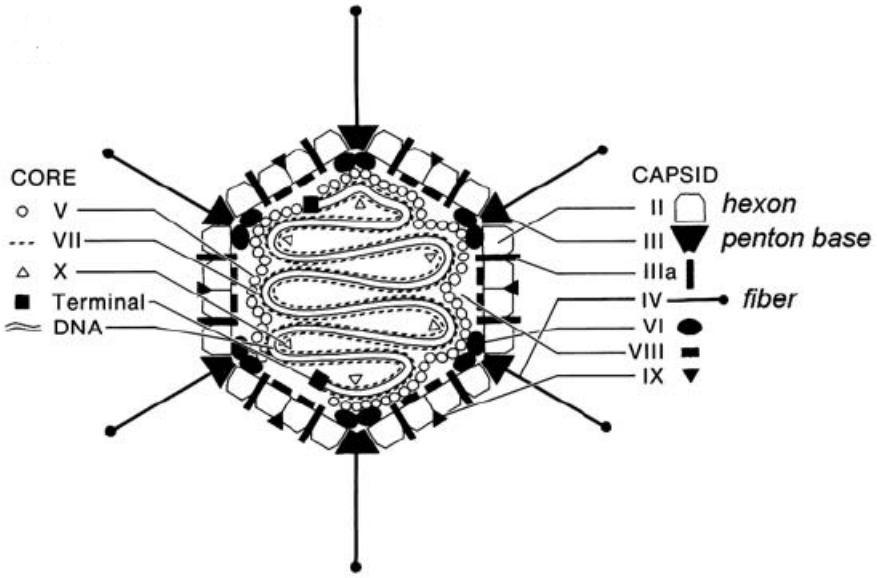


Figure 1.17: Schematic diagram of adenovirus structure
(Source: Rux & Burnett, 2004)

In general, the HAdV genome is flanked by cis-acting inverted terminal repeats (ITRs) which are required for viral DNA replication. Located near the left ITR is the cis-acting packaging signal (Ψ) which is required for the encapsidation of the Ad genome (Figure 1.18). The HAdV genome can be roughly divided into two sets of genes: the early region genes, E1A, E1B, E2, E3 and E4 which are expressed before DNA replication and the late region genes, L1 to L5 which are expressed to high levels after initiation of DNA replication. The E1A transcription unit is the first early region to be expressed during viral infection which is involved in transcriptional regulation of the virus and stimulation of the host cell to enter an S phase-like state. The E1B proteins are necessary for blocking host mRNA transport, stimulating viral mRNA transport and blocking E1A-induced apoptosis. The E2 region encodes proteins required for viral DNA replication and was divided into two

subregions: E2a encodes the 72-kD DNA-binding protein and E2b encodes the viral DNA polymerase and terminal protein precursor (pTP). The E3 region encodes at least seven proteins most of which involved in host immune evasion. The E4 region encodes at least six proteins which facilitate DNA replication, enhance late gene expression and decrease host protein synthesis. The late region genes are expressed from a common major late promoter (MLP). Most of the late mRNAs encode virion structural proteins. Four other small transcripts are also produced in addition to the early and late region genes. The gene encoding protein IX (pIX) is collinear with E1B but uses a different promoter and is expressed at an intermediate time, as is the HPIVa2 gene. Other late transcripts include the RNA polymerase III transcribed VA RNA I and II (Rosewell *et al.*, 2011).

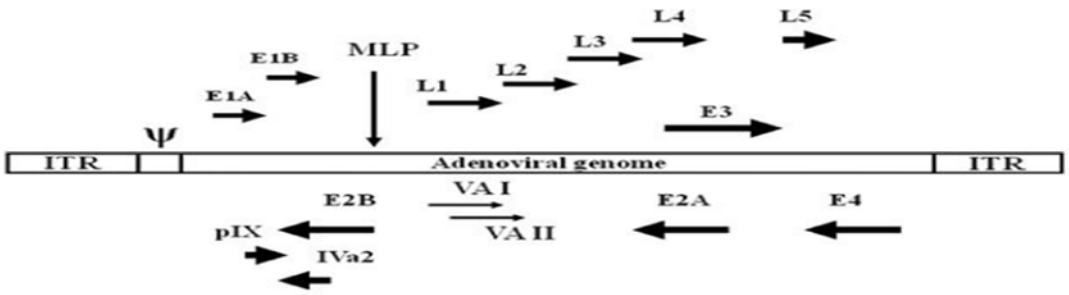


Figure 1.18: Transcription map of human adenovirus serotype 5. The genome is divided into four early region transcription units, E1-E4, and five families of late mRNA, L1-L5, which are alternative splice products of a common late transcript expressed from the major late promoter (MLP). Four smaller transcripts, pIX, Iva, and VA RNA's I and II are also produced. The 103 bp inverted terminal repeats (ITRs) are located at the termini of the genome and are involved in viral DNA replication, and the packaging signal (Ψ) located at the left end is involved in packaging of the genome into virion capsids.
(Source: Adapted from Rosewell *et al.*, 2011)

1.4.1.8.2 Human adenovirus classification

To date, there are 70 human adenovirus types classified into seven species (Human adenovirus A to G) according to their biophysical and biochemical properties. HAdV species

B is further subdivided into B1 which includes HAdV type 3, 7, 16, 21, 50 and B2 comprising of HAdV types 11, 14, 34, 35, and 55 (Russell, 2009). Previous HAdV serotype is distinguished by its resistance to neutralization by antisera to other known adenovirus serotypes. As the serological type definition is being replaced by genomic and bioinformatics criteria, the International Committee on Taxonomy viruses (ICTV) have recognized several new HAdV types (HAdV-52-HAdV-70) based on these criteria (Liu *et al.*, 2011; Matsushima *et al.*, 2012, 2013; Seto *et al.*, 2011; Walsh *et al.*, 2011).Table 1.13 depicts the current human adenovirus classification.

Table 1.13: Human adenovirus classification

	Serotypes of Human adenovirus
Human adenovirus A	12, 18, 31,61
Human adenovirus B	3, 7, 11, 14, 16, 21, 34, 35, 50, 55, 66, 68
Human adenovirus C	1, 2, 5, 6, 57
Human adenovirus D	8, 9, 10, 13, 15, 17, 19, 20, 22-30, 32, 33, 36-39, 42-49, 51, 53, 54, 56, 58, 59, 60, 62, 63, 64,65, 67, 69, 70
Human adenovirus E	4
Human adenovirus F	40, 41
Human adenovirus G	52

Source: HAdV Working Group (2016)

1.4.1.8.3 Epidemiology and seasonality

HAdV infections account for at least 5% to 15% of upper respiratory infections and 5% of lower respiratory infections in pediatric respiratory infections (Tebruegge & Curtis, 2012). Epidemiological data suggest that the majority of adenovirus infections occur in the first five years of life with a peak incidence during the first two years (Hong *et al.*, 2001; Pacini *et al.*, 1987).

HAdV infections occur throughout the year with no clear seasonal variation (Chiu *et al.*, 2010; Lee *et al.*, 2010a) although they have been reported to occur more commonly in temperate countries from late winter to early summer (Lewis *et al.*, 2009). Each species of adenovirus has been associated with different spectra of diseases and severity. The prevalence of different serotypes is known to vary geographically and seasonally, with HAdV often endemic in overcrowded spaces such as pediatric ward and military training camps (Finn *et al.*, 1988; Jeon *et al.*, 2007). Species B, C and E are usually associated with respiratory infections whereas the other species are associated with gastrointestinal, genitourinary and ocular infections (Sharma *et al.*, 2009). Species B (types 3,7,11, and 16) and species C (types 1, 2, 5, and 6) are found predominantly in children (Kidd *et al.*, 1996; Kim *et al.*, 2003) . Species B and E have been reported to cause acute respiratory tract infections in military camps (Echavarria *et al.*, 2003; Erdman *et al.*, 2002; Kolavic-Gray *et al.*, 2002). Several other studies have also showed that respiratory disease is usually caused by viruses belonging to species B1, C or E (Heim *et al.*, 2003; Schmitz *et al.*, 1983). Table 1.14 illustrates the disease conditions associated with particular species and serotypes of HAdV.

Table 1.14: Classification of human adenoviruses into species and serotype specific to various infections.

Species	**Infection**	**Serotypes**
A	Meningitis	12, 18, 31,61
B	Respiratory, ocular and renal urinary tract	3, 7, 11, 14, 16, 21, 34, 35, 50, 55, 66, 68
C	Respiratory, Hepatitis	1, 2, 5, 6, 57
D	Ocular, Meningitis	8, 9, 10, 13, 15, 17, 19, 20, 22-30, 32, 33, 36-39, 42-49, 51, 53, 54, 56, 58, 59, 60, 62, 63, 64,65, 67, 69, 70
E	Respiratory	4
F	Gastrointestinal	40, 41
G	Gastrointestinal	52

(Source: Adapted and updated from Sharma *et al.*, 2009)

Serotype identification is of utmost importance for epidemiological surveillance, detection of new strains and understanding of HAdV pathogenesis. With the advancement of molecular methods, rapid identification of different adenovirus serotypes based on the nucleotide sequencing of adenovirus genome can be done. Almost all adenovirus serotypes with the exception of serotypes 15 and 17 can be identified by PCR and nucleotide sequencing of adenovirus hyper-variable region of hexon gene (Lu & Erdman, 2006). HAdV have also been identified using virus-associated (VA) RNA gene (Kidd & Tiemessen, 1993), the polypeptide IX gene (Akalu *et al.*, 1998) or the penton and fiber genes (Xu *et al.*, 2000). Different genome types within serotypes have also been identified by restriction enzyme analysis and multiplex PCR techniques (Adhikary *et al.*, 2004; Erdman *et al.*, 2002).

Serotypes 1 through 7 account for more than 80% of HAdV infections in infants and children (Lee *et al.*, 2010b). In Asia, HAdV-3 and HAdV-7 have been the predominant serotypes associated with RTI in children (Kim *et al.*, 2003; Lin *et al.*, 2004; Zheng *et al.*, 1994). Serotypes 1 and 2 are common causes of epidemic febrile respiratory infections worldwide but appear to be less virulent than HAdV-7 (Kajon & Wadell, 1994; Kajon *et al.*, 1996) or HAdV-3 (Hong *et al.*, 2001). HAdV-3 is among the most common serotypes implicated in HAdV infections in children and adults (Gray *et al.*, 2007; Schmitz *et al.*, 1983). HAdV-3 (along with HAdV-7) was the most common cause of HAdV RTIs in Taiwan, China and South Korea (Hong *et al.*, 2001; Lin *et al.*, 2004). HAdV-4 is a cause of sporadic infections in civilians and has been implicated in epidemics of respiratory infections or pneumonia in civilian and military populations (Kajon *et al.*, 2007; McNeill *et al.*, 2000). Globally, HAdV-7 is the third most common serotype reported to World Health Organization (WHO) from 1967 through 1976 following HAdV-1 and HAdV-2 (Schmitz *et al.*, 1983). Importantly, HAdV-7 appears to be more virulent than other serotypes (Kim *et*

al., 2003). HAdV-7 infections manifest as febrile respiratory illness, pharyngoconjunctival fever, bronchitis, necrotizing bronchiolitis or pneumonia (Erdman *et al.*, 2002; Kajon *et al.*, 1996).

At least 27 genome types of HAdV-7 have been identified by enzyme restriction fragment analysis (Gerber *et al.*, 2001). HadV-7c and 7b were the predominant subtypes in the 1960s and 1970s. During this period of time, HAdV-7c was detected in South Africa, HAdV-7d in China, HAdV-7e in Brazil and HAdV-7f in Australia (Wadell *et al.*, 1985). From 1984 to 1988, a new strain of HAdV-7h was reported in Buenos Aires. In Beijing, HAdV-7d was the predominant HAdV-7 subtype responsible for HAdV pneumonia from 1980 to 1990 (Zheng *et al.*, 1994). In Taiwan, HAdV-7a was detected in 1983 but all clinical isolates from 1999 to 2001 was HAdV-7b (Lin *et al.*, 2004).

1.4.1.8.4 Clinical presentation

The main target for HAdV is the respiratory tract. Fever, pharyngitis, tonsillitis, cough and sore throat are common symptoms in children with HAdV RTI. (Tabain *et al.*, 2012). Adenovirus infections occur primarily in infants and children <5 years of age and account for 2% to 5% of respiratory illnesses among pediatric patients and 4% to 10% of childhood pneumonia cases (Hung *et al*., 2015). In immunocompromised persons, dissemination or severe respiratory failure may develop in 10 to 30% of cases (Echavarría, 2008; Kim *et al.*, 2007). Fatality rates for severe HAdV pneumonia may exceed 50%. Long-term sequelae of HAdV RTI in children include bronchiectasis, bronchiolitis obliterans, and hyperlucent lung (Edmond *et al.*, 2012; Herbert *et al.*, 1977).

HAdV infections can also cause epidemic keratoconjunctivitis (EKC), pharyngoconjunctival fever, and non-specific conjunctivitis. HAdV-8, 19, 37 and 5 are the most common serotypes associated with EKC, but other serotypes such as HAdV-3, 4, 7, 11 and 14 can cause conjunctivitis (Ariga *et al.*, 2004; Burns & Potter, 1976; Kaneko *et al.*, 2009; Schmidt *et al.*, 1975).

Gastrointestinal manifestations can be seen in HAdV infections even when the primary site of involvement is the respiratory tract. HAdV-40 and 41 are most commonly associated with GI involvement with predominant symptoms of gastroenteritis or diarrhea (Uhnoo *et al.*, 1984). Besides that, HAdV may cause urinary tract infections (UTIs) particularly among hematopoietic stem cell transplant and solid organ transplant recipients. Rare manifestations of HAdV infections include myocarditis and cardiomyopathy, encephalitis, pulmonary dysplasia and sudden infant death (Lynch *et al.*, 2011).

1.4.1.8.5 Treatment and prevention

Treatment of HAdV is usually supportive since no antiviral drug has been approved to treat HAdV although the USA did produce one for military personnel which is the oral vaccines against HAdV types 4 and 7 developed in 1971 but the supplies were depleted and vaccination was suspended by 1999 (Russell *et al.*, 2006). A new vaccine for HAdV-4 and 7 has been developed and shown to induce a good immune response in the study population. Expanded trials for safety and efficacy are in progress (Lyons *et al.*, 2008). Cidofovir is an anti-viral drug that has been shown to be effective against infection, however, due to its side effects which include nephrotoxicity, it is used sparingly in disseminated disease or those undergoing stem cell transplantation and careful monitoring of renal

function is critical (Yusuf *et al.*, 2006). Ganciclovir, vidarabine and ribavirin have inconsistent *in vitro* activity against adenovirus. In Malaysia, ribavirin and IVIG were used in patients with severe adenovirus ARTI and there are case reports of successful treatment with both although data suggests efficacy only to serogroup C adenoviruses (Foong Ng *et al.*, 2015; Emovon, 2003; Sabroe *et al.*, 1995).

1.4.1.9 Human bocavirus (HBoV)

Human bocavirus (HBoV) was first described in 2005 by Allander and coworkers in Sweden. It was detected significantly more often in the absence of other detected respiratory viruses suggesting that HBoV may be a causative agent of previously unexplained respiratory tract disease (Allander *et al.*, 2005). Since then, the worldwide presence of HBoV in children with ARTI has been confirmed (Zeng *et al.*, 2010).

1.4.1.9.1 Virus properties

HBoV is a small, non-enveloped virus which consists of isometric nucleocapsids with diameters of 18 to 26nm. It contains a single molecule of linear, negative-sense or positive-sense, single-stranded DNA with approximately 4000 to 6000 nucleotides. The genome contains three proposed open reading frames with two open reading frames putatively encoding the non-structural proteins (NS1 and NP-1) and one encoding two viral capsid proteins, VP1 and VP2 with the VP2 sequence nested within VP1 (Figure 1.19) (Allander *et al.*, 2005). The function of the HBoV NS1 protein in unknown but speculated that it plays a role in DNA replication. NP-1 is absent from other parvoviruses and its function is unknown.

The structural proteins VP1and VP2 showed high variability compared to coding sequences for the non-structural NS1 and NP-1 proteins (Schildgen *et al.*, 2008).

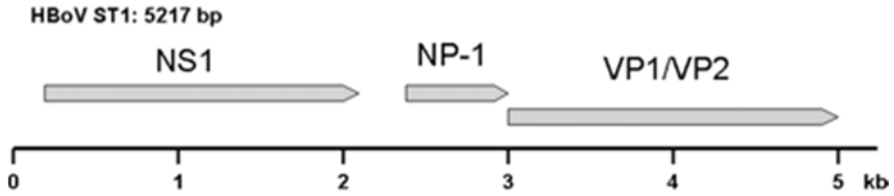


Figure 1.19: Map of HBoV genome. Schematic map of HBoV showing the three ORFs as arrows : NS1, 1920 nt, NP-1, 660 nt, and VP1/VP2, 2016 nt.
Source: (Allander *et al.*, 2005)

1.4.1.9.2 Human bocavirus classification

HBoV is a member of the family *Parvoviridae*, subfamily of *Parvovirinae* in the genus Bocavirus. HBoV was classified as a bocavirus based on genomic structure and amino acid sequence similarity shared between bovine parvovirus and canine minute virus and was thus named bocavirus (Bo for Bovine and CA for Canine) (Schwartz *et al.*, 2002). Variation in DNA can further sub-divide HBoV into 4 subtypes (HBoV 1-4) (Schildgen *et al.*, 2012). HBoV-1 detection rate has varied from 2-19% in patients with upper or lower respiratory disease (Allander *et al.*, 2005; Lau *et al.*, 2007). HBoV-2, HBoV-3 and HBoV-4 have mainly been detected in stool samples with HBoV-2 and HBoV-3 possibly being associated with gastroenteritis (Jartti *et al.*, 2012; Lee *et al.*, 2007a).

1.4.1.9.3 Epidemiology and seasonality

Reports suggest that HBoV has worldwide endemicity. Many countries such as Sweden, Australia, the United States, Japan, Germany, South Africa, Jordan, France, Canada, Iran, Spain , Netherland, Korea, Thailand, Switzerland and China have reported cases of HBoV over several years (Allander *et al.*, 2007; Arnold *et al.*, 2006; Choi *et al.*, 2006; Foulongne *et al.*, 2006; Fry *et al.*, 2007; Kaplan *et al.*, 2006; Kesebir *et al.*, 2006). Several studies have shown that HBoV is associated with ARTI in young children, with a reported worldwide prevalence ranging between 1.5 to 19% (Allander *et al.*, 2007; Bastien *et al.*, 2006; Chung *et al.*, 2006). Primary infection with HBoV might occur early in life and children between the ages of six and 24 months seem to be mostly affected but older children can be infected too (Chung *et al.*, 2006; Naghipour *et al.*, 2007; Sloots *et al.*, 2006).

In a study of human bocavirus infection from all age groups in Canada, no seasonal prevalence was observed for HBoV infection, the virus was found throughout the year. This might be due to low prevalence in the study. 89% of hospitalizations were in persons ≤ 5 years of age, which suggests that HBoV may cause more severe respiratory illness in infants and children, similar to disease caused by RSV, HMPV, HCoV-NL63 and other respiratory illness (Bastien *et al.*, 2006).

In Malaysia, the first case of HBoV was reported in 2011 in a 13 month old boy diagnosed with pneumonia and asthma (Etemadi *et al.*, 2012). There was not much report on HBoV prevalence done in Malaysia other than a study done by Rogayah and colleagues in 2012 in which HBoV was detected in 5.6% (7/125) of children ≤ 3 years of age. Phylogenetic analysis showed that they belong to HBoV1 and were highly homologous to strains detected from other countries (Rogayah *et al.*, 2014).

HBoV positive ARTIs occur in children across a range of months. There is no clear seasonality demonstrated. However, HBoV has been detected mostly in autumn and winter months (Garcia *et al.*, 2009; Smuts & Hardie, 2006). A study done in Italy could not confirm a seasonal distribution of HBoV infections in their study of hospitalized infants with RTI (Maggi *et al.*, 2007). Some studies done with most specimens collected during the winter months which are the epidemic season for most viral RTIs. Therefore, the numbers reported in most studies probably reflect a mix of incidence and carrier prevalence. The true incidence and seasonality of primary HBoV infection remain unknown (Schildgen *et al.*, 2008).

1.4.1.9.4 Clinical presentation

HBoV was first identified in respiratory samples, thus it has been suggested as a respiratory infectious agent (Allander *et al.*, 2005). Clinical symptoms commonly associated with HBoV infection include hypoxia, fever, pneumonia, bronchiolitis, asthma and wheezing (Allander *et al.*, 2007; Monteny *et al.*, 2007; Völz *et al.*, 2007). HBoV is also detectable in asymptomatic controls but with a lower incidence. For example, HBoV was detected in 17% of children hospitalized because of respiratory infection, while only 5% of the surveyed asymptomatic children were HBoV positive (García-García *et al.*, 2008). In Thailand, HBoV was identified in 3.9% of children with LRTI and 1% of asymptomatic children (Fry *et al.*, 2007). Case reports worldwide suggest that HBoV is most often associated with upper and lower respiratory tract infections. Among those of symptomatic children studies, about one-quarter have upper respiratory tract infections, and a variable range from 0 to 76% have had lower respiratory tract involvement. The high occurrence of lower respiratory symptoms,

especially wheezing had led to speculation that HBoV may be a significant cause of asthma exacerbations (Milder & Arnold, 2009).

In addition, several studies detected HBoV in stool specimens from children with acute gastrointestinal illness. A high rate of detection of co-pathogens in the stool (56-58%) was also reported (Albuquerque *et al.*, 2007; Lau *et al.*, 2007; Lee *et al.*, 2007a). However, the role of HBoV infection in gastrointestinal tract is still unclear.

Cases of HBoV identified in immunocompromised patients have been described. The symptoms occurring in such cases include fever, upper and lower respiratory tract symptoms, gastrointestinal symptoms, seizures, and hepatitis. The role of HBoV as a sole pathogen in such cases is difficult to prove beyond doubt (Milder & Arnold, 2009).

1.4.1.9.5 Treatment and prevention

There is currently no specific approved treatment for HBoV infection. Due to the lack of cultivability and high frequency of detection as a co-pathogen, besides its relatively benign course in most patients, specific treatments will not likely be available soon. Currently, there have been no studies on the benefits of particular therapeutic approaches for HBoV-infected individuals and no proposals for novel therapeutics. Symptomatic treatment may be required in severe cases and is analogous to the treatment of other respiratory tract infections (Schildgen, 2013).

1.4.2 Bacterial infections

Besides viral causes, bacterial infection is also an important cause of many types of ARTIs. Bacterial pneumonia is often caused by *Streptococcus pneumoniae* (pneumococcus) or *Haemophilus influenzae*, mostly type b (Hib), and occasionally by *Staphylococcus aureus* or other streptococci. Other pathogens, such as *Mycoplasma pneumoniae* and *Chlamydia pneumoniae*, cause atypical pneumonias.

The most common bacteria detected in paediatric airways is *Streptococcus pneumonia*e (pneumococcus) which accounts for about two-thirds of cases of bacterial pneumonia and is the most common cause of pneumonia leading to hospitalization. Pneumococcal bacteremia is most common in children aged 3-36 months, an age at which children no longer have maternal IgG but have not yet developed their own protective antibodies. Pneumococcus is reported as a common cause of bacterial meningitis and bacterial pneumonia in US children younger than 2 years (Robinson *et al.*, 2001). Development of the conjugate vaccine which target different serotypes of pneumococcus has been developed and its utilization worldwide have reduced mortality (O'Brien *et al.*, 2009).

Hib is also a common bacterial pathogen involved in paediatric ARTIs after pnuemococcus. Before the introduction of vaccines, Hib was the leading cause of bacterial meningitis and other invasive bacterial disease such as epiglottitis and sinusitis among children younger than 5 years of age with approximately two-thirds of all cases occurred among children younger than 18 months of age. Hib caused meningitis in 50-65% of cases in prevaccine era. With the development of a Hib conjugate vaccine since the 1990s,

vaccination has greatly reduced the paediatric mortality rate (Centers for Disease Control and Prevention, 2015).

Infections such as the fungi *Pneumocycstis jirovecii* or the bacteria *Mycoplasma pneumoniae*, *Chlamydia trachomatis* and *Chlamydophila pneumoniae* have all been shown to be involved with paediatric ARTIs with comparatively less known than the two most common pathogens. The impact of these atypical bacterial pathogens varies in different studies on ARTIs and also with conditions the child may have such as HIV (Scott *et al.*, 2008).

1.5 Overview of pathogenesis

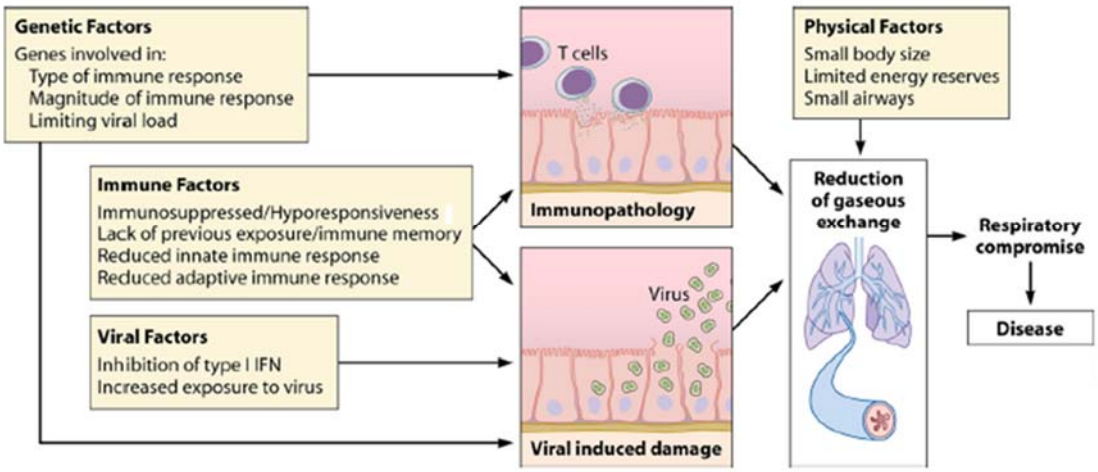


Figure 1.20: Interplay of factors that cause disease following respiratory viral infection and impact of infancy.
Source: Adapted from (Tregoning & Schwarze, 2010)

Young age acts as a metafactor reflecting the interplay of factors causing disease following viral infection (Figure 1.20). Young children are more vulnerable to respiratory infection due to the small body size of child, particularly airway size, transmission dynamics as a result of multiple close contacts between small children. Furthermore, younger children

have smaller energy reserves and are more likely to get exhausted by the effort of breathing which may ultimately cause bronchiolitis (Tregoning & Schwarze, 2010).

Genetic risk factors have also been identified contributing to respiratory viral disease damage. For example, polymorphisms in surfactant protein-A (SP-A) were associated with an increased risk of RSV bronchiolitis (Löfgren *et al.*, 2002) and SP-A deficiency might increase RSV viral load (Levine *et al.*, 1999). Deficiency of type I interferon (IFN) (Wark *et al.*, 2005) and type III IFN (Contoli *et al.*, 2006) production in bronchial epithelial cells from asthmatics increased the risk of severe viral infection.

The infant immune system is different from the adult immune system and this has a critical impact on susceptibility of respiratory viral infection. In children, there is a general naivety of their immune system which includes the lack of prior exposure to pathogens that leads to a lack of immune memory. There is also a tendency toward hyporesponsive immune responses in early life, characterized by both reduced innate and adaptive immune responses (Adkins *et al.*, 2004). Studies have demonstrated that there are reduced responses to innate stimuli. The level of the response may depend upon the effector molecule that is being used as a readout: the level of interleukin-6 (IL-6) appears to be increased (Angelone *et al.*, 2006), while the level of tumor necrosis factor (TNF) (Levy *et al.*, 2004) or type I IFNs (De Wit *et al.*, 2004) is decreased. The downstream consequence of the failure to initiate type I IFN responses causes minimal dendritic cell (DC) activation in the adaptive immune response in infants. Dendritic cell immaturity may lead to the reported skewing of the immune response to a Th2 phenotype and also influence the strength of B-cell responses. In addition, both CD4 and CD8 functions were reported to be deficient, which may in turn lead to reduced viral clearance and increased reinfection (Tregoning & Schwarze, 2010).

The initial detection of viruses by the immune system is vital for viral control. Host receptors recognize basic components of viruses triggering an immune response. These viral components, termed pathogen-associated molecular patterns (PAMPs), are constituents of the virus that cannot be evolved away from. Respiratory viruses are detected through the innate immune system extracellularly by Toll-like receptor (TLR2), TLR4, TLR6, in the endosome by TLR3, TLR7 and TLR9, and in the cytoplasm by RIG-1 (retinoic acid-inducible gene I), MDA-5 (melanoma differentiation-associated gene 5), and NLRP3 (NLR family, pyrin domain-containing 3). For example, RSV was shown to interact with the TLR4. TLR3 recognizes double stranded RNA, TLR7 recognized single-stranded RNA and TLR9 recognizes unmethylated CpG repeats (Tregoning & Schwarze, 2010).

The type I interferon (IFN) system is critical for the host defence against virus, and evading it is of critical importance to all viruses. All respiratory viruses have mechanisms to avoid the type I IFN response. Viruses can hide their PAMPs. For example, the influenza virus NS1 protein conceals the viral genome from detection. Both influenza virus and RSV target RIG-I and RV targets MDA5 (Barral *et al.*, 2007; Ling *et al.*, 2009; Pichlmair *et al.*, 2006). Downstream signalling to these receptors can be inhibited. RSV was shown to inhibit IFN production. SARS-CoV was shown to block NF-kB function. Besides that, HRV, RSV, SARS-CoV and HPIV can inhibit the activation of IRF3 (Kopecky-Bromberg*et al.*, 2007; Kotla *et al.*, 2008; Spann *et al.*, 2005).

The adaptive immune response is principally evaded by mutation of viral proteins. Large number of circulating viruses such as over 110 serologically distinct HRVs and the segmented genome of influenza virus increases its ability to rapidly change genotypes. Thus, the memory response that it induces may be poor (Tregoning & Schwarze, 2010).

In conclusion, viruses have evolved to evade the immune system, and this immune evasion is critical for viral host-specificity and has an important impact on the host response to infection. The combination of viral immunosuppression and the hyporesponsiveness of the early-life immune response may increase the amount of virally mediated damage and therefore increase likelihood of disease (Tregoning & Schwarze, 2010).

1.6 Diagnostic methods for acute respiratory infections

1.6.1 Upper and lower airway samples

Respiratory viruses are usually identified in samples from upper respiratory tract. All nasopharyngeal sample types, including nasopharyngeal aspirates (NPA), washes, swabs, appear to allow for equally sensitive PCR analysis (Chan *et al.*, 2008; Munywoki *et al.*, 2011; Spyridaki *et al.*, 2009). However, to what extent upper airway virus findings reflect true viral infections in the lower airways has been issues that have not been adequately resolved. Paediatric studies using bronchoalveolar lavage (BAL) or lung biopsy samples have indicated that many respiratory viruses seem to penetrate the distal bronchioles and can be demonstrated at similar detection rates in upper and lower airways (Malmström *et al.*, 2006; Simons *et al.*, 2005).

HRV, the most frequent causative agent of the common cold, has been presumed to occur mainly in the upper airways and it is also associated closely with asthma exacerbation. (Khetsuriani *et al.*, 2007). Indeed, HRV has been found by *in situ* hybridization in lower bronchial biopsy specimens of 45% of young children with recurrent asthma-like symptoms (Malmström *et al.*, 2006). In another study, HRV was detected by PCR both in nasal and

tracheal secretions (Simons *et al.*, 2005). HRV replicates well at lower airway temperatures *in vitro* (Papadopoulos *et al.*, 1999).

Sputum inductions are shown to increase the sensitivity and specificity of diagnosis of lower airway infections. However, most of the pathogenic viruses were often identified also in samples from upper airways (Lahti *et al.*, 2009). Although sputum induction does provide good quality samples from the lower respiratory tracts, it is not recommended for routine use in children with LRTI since it is an unpleasant procedure and does not usually provide unique or additional virological information.

Generally, respiratory viruses initiate the infection from the upper respiratory tract epithelium. The infection then spreads to the lower respiratory tract after a replication period of the viruses from the epithelium in upper respiratory tract and this is followed by different courses of the lower respiratory tract infections (Stensballe *et al.*, 2002; Wat, 2004). Following the pathogenesis of these infections, nasal samples may be used for diagnosis from different clinical forms of viral ALRI (Heikkinen *et al.*, 2002; Stensballe *et al.*, 2002). However, when using nasal samples, the phase of infection may be an important factor affecting the sensitivity of the methods used. All ALRI cases evaluated should be in the acute phase of the infection. It may be impossible to detect the viruses in the nasal mucosa after one week of the initial symptoms because of the possible transportation of the agent to the lower respiratory tract (Bulut. *et al.*, 2007).

Recently, a flocked-nasopharyngeal swab (NFS) was designed to improve the yield of nasopharyngeal epithelial cells and enhancing diagnostic yield (Copan Diagnostics, Corona, CA). Studies showed that flocked swabs yielded more cells and provided better clinical specimen when compared with traditional rayon nasal swab or Dacron swabs with

less patient discomfort (Daley *et al.*, 2006; Dube *et al.*, 2013). When comparing NFS with NPAs for rapid diagnosis of respiratory viruses in children, Chan *et al.* (2008) found that both NPA and NFS were comparable for detecting influenza virus by reverse transcription –PCR (RT-PCR) but an NPA was slightly more sensitive than NFS for detecting RSV. They concluded although NFS has a slightly lower sensitivity compared to NPA, the NFS is still a suitable alternative for rapid respiratory diagnosis by antigen detection or RT-PCR. In addition, the collection of NFS was less invasive for children compared with NPA besides NFS collection may generate less aerosol associated cross infection hazard.

1.6.2 Laboratory diagnosis

Over the past decade, virus isolation, antigen detection and serology have been used for diagnosing respiratory virus infections. The diagnostics of respiratory viral infections began in 1933 by the discovery of the influenza A virus (Jartti *et al.*, 2013). Since then, several other major respiratory viruses were discovered, including the RSV, HRV, HAdV, EV, HPIV and HCoV. These methods yielded an overall virus detection rate of only less than 40% in children with respiratory illnesses (Heiskanen-Kosma *et al.*, 1998; Jennings *et al.*, 1987). In the 1990s, a new era began with the development of molecular amplification-based techniques (polymerase chain reaction-PCR) which enabled the discovery of new respiratory viruses in the respiratory tract, including HMPV, new HCoV, HBoV, HRV-C and polyomaviruses (Jartti *et al.*, 2011).

1.6.2.1 Antigen detection tests

Rapid immunoassays are widely used for the detection of influenza viruses and RSV. Rapid immunoassay formats include membrane-based enzyme immunoassay, lateral flow immunochromatography, and optical immunoassay. The sensitivity of rapid RSV immunoassays in pediatric patients is generally higher than that of cell culture due to the lability of RSV (Ginocchio, 2007). Evaluations of rapid immunoassays in 2009 for detection of the novel 2009 influenza A (H1N1) virus have demonstrated variable and generally poor sensitivity, in some cases 25% or lower compared to reverse transcriptase-PCR (Faix *et al.*, 2009; Ginocchio *et al.*, 2009). However, with the resurgence of the influenza A (Flu A; subtype H1N1) pandemic 2009 (pdm09) strain in the years 2013 and 2014, immunoassays are now better at detecting influenza (Arbefeville *et al.,* 2015). Enzyme-linked immunosorbent assay (ELISA) are also available for detection of respiratory viruses. ELISA can detect viral antigens that are suspended in solution or associated with inflammatory material (Henrickson & Hall, 2007).

Another example of antigen detection includes immunofluorescence testing of nasopharyngeal aspirates for respiratory viruses. The main advantage of these assays is that they are rapid to perform with the result being available within a few hours. Direct fluorescent antibody (DFA) staining of viral antigens in patient specimens is generally considered to be more sensitive than rapid immunoassays (Aslanzadeh *et al.*, 2008). DFA reagents have long been available for detection and identification of influenza A and B viruses, RSV, HPIVs and HAdV. Recently, DFA also showed high sensitivity of HMPV detection on nasopharyngeal aspirates from children (Vinh *et al.*, 2008). DFA was substantially more sensitive than rapid immunoassays for detection of the novel 2009

influenza A virus though less sensitive than virus isolation or RT-PCR (Ganzenmueller *et al.*, 2010).

A relatively new adaptation of antigen detection technique involves using flow cytometry (FCM). However, due to the small size of viruses, they cannot be detected directly using FCM. It can be detected indirectly in infected cells in clinical samples or after inoculation and culture of viruses in cell culture. FCM detects and quantifies viral antigens on the surface of, or within, infected cells. This application of FCM has been used for several viruses, including HIV, cytomegalovirus as well as influenza virus (Yan *et al.*, 2005).

1.6.2.2 Conventional culture techniques

Many clinically significant viruses replicate *in vitro*, and virus recovery correlates well with acute disease. Virus isolation in cell cultures has long served as the "gold standard" for virus detection and it is the method to which all others have been compared before the introduction of molecular diagnostics (Hsiung, 1984).

Almost all viral diagnostic labs that attempt to cultivate respiratory viruses utilize cell culture. The choice of cell line(s) to be used depends upon the virus(es) of interest. Unfortunately, there is no single cell line that will support the growth of all respiratory viruses (Table 1. 15), and some viruses grow poorly (HMPV, HCoV-229E, HCoV-NL63) or not at all in cell culture (HCoV-OC43 and HKU1, HBoV, HRhV-C). However, cells from different sources have been co-cultivated to increase the spectrum of virus strains detected. One such commercially available cell mixture is the R-mix FreshCells line (Diagnostic Hybrids, Athens, OH), a combination of mink lung cells (Mv1Lu) and human adenocarcinoma cells (A549). The R-mix cells have similar sensitivities to those observed

with individual cell lines for detection of influenza A and B viruses, RSV, HPIVs, and HAdV (Barenfanger *et al.*, 2001; Weinberg *et al.*, 2004).

Culture conditions need to be modified to allow the growth of certain viruses. For example, exogenous protease must be added (TPCK-trypsin) to support viral growth in continuous cell lines such as MDCK as they do not produce proteases needed to cleave the hemagglutinin of influenza viruses. The presence of exogenous protein, such as fetal bovine serum, can inactivate the protease and prevent growth, so FBS-containing media needs to be removed and cells rinsed with FBS-free media prior to addition of trypsin-containing media. Some rhinoviruses grow better at lower temperatures, so incubation of cultures at 33-34˚C instead of 36-37 ˚C can improve the recovery of rhinoviruses (Atmar & Greenberg, 2010).

A variety of methods are used to monitor cell culture once they are inoculated. Visual inspection for cytopathic effect (CPE) can identify changes characteristic of virus replication, but confirmatory test (such as IF or EIA or RT-PCR) need to be performed for final virus identification. Evaluation for the presence of hemadsorption of red blood cells (RBCs) or hemagglutination activity of the culture supernatant can be performed every two to three days to identify early replication of the hemadsorbing influenza or HPIVs.

A disadvantage of these approaches is that it can take up to two weeks for a virus to grow to detectable levels in cell culture. Shell vial assays have been developed to overcome this problem which allows earlier detection of respiratory viruses in cell culture. The clinical sample is inoculated onto the cell monolayer by low-speed centrifugation, and after 24-48 hours of incubation, the cell line is fixed and stained with labelled, virus-specific antibodies. This approach can achieve comparable sensitivity to standard cell culture but it has the

disadvantage of not providing virus for further characterization such as antigenic analysis or antiviral testing.

Table 1.15: Preferred cell lines for virus isolation

Virus	**Cell line**
Rhinovirus	Human embryonic lung: WI38, MRc-5, RD
HCoV-229E HCoV-NL63 HCoV-SARS	MRC-5 LLC-MK2, Vero-B4 Vero E6
Adenovirus	Human embryonic kidney, A549, HeLa, Hep-2, KB, 293
Influenza virus A and B	Primary monkey kidney cells, Madin-Darby canine kidney (MDCK), LLC-MK2
Parainfluenza virus	Primary monkey kidney cells, LLC-MK2
Respiratory syncytial virus	Human heteroploid cells: Hep-2, HeLa, A549
Human metapneumovirus	LLC-MK2

(Source: Adapted from Atmar and Greenberg, 2010).

1.6.2.3 Serology

Serologic methods can be used to identify respiratory virus infections, but are rarely used for clinical diagnosis because they require the evaluation of paired sera (acute and convalescent phase) collected at least two weeks apart to identify either seroconversion or a fourfold rise in antibody titer. Diagnosis cannot reliably be made with a single serum sample. Thus, these methods are usually used for epidemiologic and other clinical research studies. There are a variety of methods that can be used, and these include complement fixation (CF), enzyme-linked immunosorbent assays (ELISAs), hemaagglutination inhibition (HAI) and neutralization assays.

Complement fixation (CF) assays have been developed for influenza viruses, RSV, HPIVs, HAdV, HCoV-229E, -OC43. These assays target genus- or type-specific epitopes. CF assays are not used for rhinoviruses and enteroviruses because of heterotypic responses

and lack of correlation of seroresponse with those measured by neutralization methods. CF assays have not been described for the more recently discovered viruses, such as HMPV and the new coronaviruses.

Enzyme-linked immunosorbent assays (ELISAs) are among the most flexible of the serologic assays. The antigen(s) used in these assays can range from whole virus to viral proteins. ELISAs have been applied to measuring antibody responses not only in serum samples but also in respiratory secretions. They can also be modified to measure specific immunoglobulin (IgG, IgA, IgM) responses after infection or vaccination (Belshe *et al.*, 2000; Burlington *et al.*, 1983).

Hemaagglutination inhibition (HAI) assays can be performed for viruses that have hemagglutination activity. These include influenza viruses, HPIVs, HCoV OC43, some adenoviruses and rhinoviruses, and also enteroviruses. HAI assays are most commonly performed for influenza viruses and HPIVs. The hemagglutinating proteins are not visible under light microscopy but can be detected by their affinity for erythrocytes. HAI testing is routinely performed at the end of the incubation period for cell cultures that fail to produce CPE or earlier in the incubation period, at days 3 and 7 of incubation (Minnich & Ray, 1987).

Neutralization assays are based upon the ability of serum (or respiratory secretion) to prevent virus replication, so these assays can only be performed with the cultivable viruses. Virus is mixed and incubated with dilutions of serum, and then inoculated onto the cell culture. The quantity of virus used in the assay depends on the assay format and the virus being used, but varies from < 10 TCID50 for the rhinovirus macroneutralization assays to 100 TCID50 for influenza and HPIVs. Evidence of neutralization in serum dilution also varies by assay and includes absence of CPE on the cell culture, absence of hemagglutination

activity in the culture supernatant (Frank *et al.*, 1980) and reduction in the number of plaques in the cell culture (Siber *et al.*, 1994).

1.6.2.4 Molecular techniques (Nucleic acid amplification tests)

Molecular methods are increasingly being used for the diagnosis and characterization of respiratory viruses. Reverse-transcription polymerase chain reaction (RT-PCR) assays are the most commonly used and target conserved genes within the targeted virus. Other molecular assay formats, including nucleic acid sequence-based amplification (NASBA) and loop-mediated isothermal amplification (LAMP), have also been described for some of the respiratory viruses. The main advantages of amplification of viral genetic material by PCR include that they are highly analytical, and also sensitive besides its diagnostic value.

Monoplex RT-PCR or PCR assays have been developed for every respiratory virus (Mahony, 2008). However, it was soon realized that PCR was too laborious, expensive, and sample consuming, if a sufficient number of virus analyses was to be made to be clinically meaningful. Many studies have shown viral co-infections in children with respiratory illness. (De Paulis *et al.*, 2011; Debiaggi *et al.*, 2012; Nascimento *et al.*, 2010; Tran, 2013). The problem of identifying multiple potential causes of infection has been addressed by the development of sensitive multiplex RT-PCR assays. Multiplex PCR is defined as running more than one PCR analysis in a single reaction tube. This is accomplished by applying more than one set of primers that produce amplicons of varying sizes and are specific for different, individual viral sequences (Lam *et al.*, 2007). Multiplex PCR approaches are becoming increasingly popular for the detection of respiratory viruses, since lower reagent costs and

shorter throughput time favour the cost-effectiveness of multiplex PCR over multiple single PCRs (Fox, 2007; Mahony *et al.*, 2009).

In real-time PCR, amplification (as detected by an increase in fluorescence) and analysis occur simultaneously. Real-time PCR is suitable for quantitative analysis, but currently available commercially kits for detection of respiratory virus nucleic acids have only qualitative claims. Most real-time PCR instruments are limited in the number of emission channels available for multiplexing. As a result, commercially available multiplex real-time PCR kits are generally limited to the detection of four or fewer nucleic acid targets. Real time PCR are not designed to distinguish between different subgenera or subtypes of all respiratory viruses while multiplex RT-PCR capable of distinguishing between different subgenera or subtypes of viruses by post-PCR detection methods as the amplicons can be visualized under ultraviolet light upon gel electrophoresis and be extracted for further sequencing reaction (Henrickson & Hall, 2007; Mahony, 2008).

Recently, several commercial multiplex assays for the detection respiratory viruses have been introduced; these include the ResPlex II Panel v2.0 (Qiagen, Hilden, Germany), xTAG® respiratory Viral Panel fast (Abbott, Rungis, France), Seeplex RV assay (Seegene Inc., Seoul South Korea), RespiFinder® SMART 22 (Pathofinder, Maastricht, The Netherlands), Clart® Pneumovir (Genomica, Madrid, Spain) and Fast Track Diagnostics Respiratory Pathogen 33 (Junglinster, Luxembourg)**.**

Because of the sensitivity and rapid turn-around time, nucleic acid amplification techniques such as PCR will likely be the focus of further advances in respiratory virus diagnostics. However, nucleic acid amplification test can create diagnostic conundrums due to its high sensitivity. Additional research is needed to elucidate the clinical significance of

positive PCR results that are persistent in a patient, and when two or more respiratory viruses are detected in a single specimen. Current and future diagnostic options will include antigen, molecular and culture-based methods. The performance characteristics and limitations of these methods will vary greatly with the new generations of assays. It is important that diagnostic virologists and clinicians understand these characteristics and limitations.

1.6.2.5 Molecular point-of-care methods

Rapid diagnostic tests for influenza, and other respiratory viruses, based on antigen detection in nasal samples have been available for many years where sensitivity is around 50% (Chartrand *et al*., 2012). The poor sensitivity of these antigen-based tests limits their clinical utility and their use has not been associated with a reduction in antibiotic (Nicholson *et al*., 2014).

The current gold standard diagnostic test for respiratory viruses is laboratory performed polymerase chain reaction (PCR) which is highly sensitive and specific but has turnaround times of at least 24 h and requires specialist laboratory facilities and expertise (Mackay 2007). New rapid, molecular tests have recently been developed, including the FilmArray Respiratory Panel. These molecular platforms are comparable in accuracy to laboratory PCR, without the need for specialist laboratory support and expertise, and can potentially be used as a point-of-care test (POCT), but the evidence for molecular POCT improving patient outcomes is weak (Brendish *et al*., 2017).

The FilmArray Respiratory Panel (BioFire Diagnostics, Utah, USA, owned by bioMérieux) uses nested real-time PCR to detect 20 respiratory pathogens. The FilmArray requires only 2 min of "hands on" time and produces a test result in about one hour (Brendish

et al., 2017). The FilmArray Respiratory Panel is both FDA-cleared and CE IVD marked. The viral pathogens detected by the FilmArray Respiratory Panel are: Influenza A (untyped, A/H1, A/H1-2009, A/H3), Influenza B, Adenovirus, Coronaviruses (HKU1, NL63, 229E, OC43) Human Metapneumovirus, Human Rhinovirus/Enterovirus, Parainfluenza (types 1, 2, 3, 4) and Respiratory Syncytial Virus. Three bacterial respiratory pathogens are also detected: *Bordetella pertussis, Chlamydophila pneumoniae* and *Mycoplasma pneumoniae* (Popowitch *et al*., 2013). The FilmArray respiratory panel is broadly equivalent in accuracy to laboratory PCR, and use has been validated on nose and throat swabs, nasopharyngeal aspirates, lower respiratory tract samples, and samples from immunocompromised patients (Ruggiero *et al.,* 2013).

Rapid molecular platforms, such as the FilmArray Respiratory Panel, have equivalent diagnostic accuracy to laboratory PCR and can generate a result in 1 h making them deployable as POCT. Molecular point-of-care testing for respiratory viruses in hospital has the potential to improve the detection rate of respiratory viruses, improve the use of influenza antivirals and reduce unnecessary antibiotic use, but high quality randomised trials with clinically relevant endpoints are needed (Brendish *et al.,* 2017).

1.7 Co-detection of respiratory viruses

Over the past few years, there has been an increase in the number of studies using multiplex RT-PCR to investigate the etiology and prevalence of acute respiratory infection. In some studies, viruses and bacteria have been detected in over 70% of samples (Bezerra *et al.*, 2011; Hoffmann *et al.*, 2012; Simusika *et al.*, 2015). The advance of molecular technique results in a much higher detection rates compared to traditional methods such as viral culture or serological test. It has also been reported that the rate of multiple respiratory virus detection has increased with more sensitive viral diagnostic methods (Asner *et al.*, 2015; Goka *et al.*, 2014).

Co-detection or multiple respiratory virus detection is a more appropriate terminology to use instead of co-infection. A positive PCR doesn't necessary mean that a patient is currently "infected" by the respiratory virus of interest. What we may state is that the viral RNA or DNA has been "detected" in the patient's specimen. Similarly, if we have multiple PCR positives from the one patient's specimen, we cannot say that the patient has a "co-infection", rather the results indicate "co-detection" of the viral RNA or DNA templates.

In addition, positive PCR results do not necessarily reflect active virus replication. Instead, they may reflect prolonged shedding after acute infection (e.g. HBoV), latent chronic infection (e.g. HAdV) or asymptomatic acute infection (HRV). Thus, their clinical significance during an acute episode, especially when co-detected with other pathogens, may be uncertain.

Besides, little can also be said about the clinical impact of that detection when it occurs from a patient with illness that manifests at a different anatomical site. For example, nasopharyngeal aspirates and swabs collected from upper respiratory tract of a patient with a clinical diagnosis of pneumonia or some other illness associated with the lower respiratory tract, may not alone be used to define an association between the illness and the target virus. Such an association may be suggested but until the virus is found to replicate in that particular tissue that causes a local immune response, no stronger link can be made.

Nevertheless, there were still studies focusing on the severity of virus co-detection which resulted in different findings. Table 1.16 showed the impact of viral co-detection on clinical severity in hospitalized children with respiratory illnesses. The discrepant results may be due to differences in studied population (age group, severity of diseases), viral detection method and performance characteristics of the molecular respiratory panels.

Table 1.16: Impact of viral co-detection on clinical severity in hospitalized children

Study	Setting	Population	Most common viruses identified	Co-detection	Effect of co-detection
Richard *et al.* 2008 Retrospective 2003/2004	Hospital, France	Infants < 1y, AVB short-stay unit (n=92) or PICU (n=88)	Viruses in 96.1% (PCR/IF) RSV (70.6%, 73.6%) HRV (18.5%, 25.3%)	24.4%	Increased risk of PICU (OR=2.7 [95% CI 1.2-6.2])
Cilla *et al.* 2008 Prospective 2004/2006	Hospital, Spain	Children < 3y, CAP (n=315)	Virus in 66.9% (PCR/culture) RSV 19.8% Bocavirus 14.2% HRV 13.6%	27%	Increased hospitalization (67.2% vs. 46.1%, p=0.005)
Calvo *et al.* 2008 Prospective 2000/2003	Hospital, Spain	Children < 2y, ARI (n=749)	Virus in 65.9% (PCR) RSV 35.4% HAdV 19.3% HRV13.5%	17.4%	Higher fever Longer hospitalization
Canducci *et al.* 2008 Prospective 2004/2006	Hospital, Italy	Children < 2y, ARI (n=322)	Virus in 46.6% (PCR) RSV 28% hMPV 14.3% [HRV not tested]	14%	RSV mono-infection more severe
Aberle *et al.* 2005 Prospective 2000/2004	Hospital, Austria	Infants < 1y, LRI (n = 772)	Virus in 77% (PCR) RSV 28% HRV 32%	26%	RSV coinfection with more severe clinical course
Suryadevara *et al.* 2011 Prospective 2007/2010	Hospital, USA	Children < 2y, ARI (n = 201)	Virus in 93% (PCR) RSV 58% HRV/enterovirus 33%	28%	No impact on severity
Nascimento *et al.* 2010 Prospective 2006/2007	Hospital, Brazil	Children < 2y, AVB (n = 77)	Virus in 93.5%(PCR) RSV 63.6% HRV 39%	44%	No impact on severity
Stempel *et al.* 2009 Retrospective 2003/2004	Hospital, USA	Children < 2y, AVB (n = 180)	Virus in 93% (PCR) RSV 71% Adenovirus 15% [HRV not measured]	23%	No comments

Table 1.16 continued

Study	Setting	Population	Most common viruses identified	Co-detection	Effect of co-detection
Marguet *et al.* 2009 Prospective 2002/2004	Hospital, France	Children < 1y, AVB (n = 209)	Virus in 94.7%(PCR/IF) RSV 60.3% HRV 21.5%	21.5% (RSV/HRV)	RSV infection more severe than HRV No impact of coinfection
De Paulis *et al.* 2011 Retrospective 2005	Hospital, Brazil	Children < 2y, LRI (n = 304)	Virus in 72% (PCR) RSV 80.4% Adenovirus 9.1%	25.1%	No impact On severity
Yoshida *et al.*, 2012 Cross-sectional 2007/2010	Hospital, Vietnam	Children < 5y, ARI (n= 1992)	Virus in 60.9% (PCR) HRV 24.2% RSV 20.1% IFV 12%	9.5%	RSV increases the risk for LRTI when co-infected with HRV, HMPV and PIV but not IAV.
Diaz *et al.*, 2015 Cross-sectional 2012/2013	Hospital, Mexico	Children < 5y, ARI (n= 162)	Virus in 67% (PCR) HMPV 22% HAdV 16% RSV 14%	31.6%	No impact on severity
Meligy *et al.*, 2015 Prospective 2013/2014	Hospital, Cairo	Children < 3y, LRI (n= 51)	Virus in 86.3% (PCR) HRV 72.2% PIV 27.3% RSV 20.5%	63.7%	No impact on severity

95%CI = 95% confidence interval; ARI = acute respiratory illness; AVB = acute viral bronchiolitis; CAP = community-acquired pneumonia; hMPV = human metapneumovirus; HRV = human rhinovirus; IF = immunofluorescence; LRI = lower respiratory illness; OR = odds ratio; PCR = polymerase chain reaction; PICU = pediatric intensive care unit; RSV = respiratory syncytial virus; y = year.

1.8 Statement of the research problems

Acute respiratory tract infections (ARTI) are one of the major causes of morbidity and mortality in young children throughout the world especially in developing countries (Nair *et al.*, 2013; Williams *et al.*, 2002). Majority of LRTI in developed countries have been reported to be often due to viral pathogens (Dut & Kocagöz, 2016; Mäkelä *et al.*, 1998). According to WHO (2013) in the paper "Research needs for the battle against respiratory viruses, respiratory tract infection (RTIs) is the leading cause of death worldwide. Acute lower respiratory infections (ALRI) are estimated to cause 3.9 million deaths per year, and pneumonia alone is the leading single cause of mortality in children under 5 years of age, with approximately 1.2 million children dying each year. In 2015, Ministry of Health Malaysia reported diseases of the respiratory system is the second main causes of hospitalization in the year 2014, accounting for 11.26% and 13.09% of hospitalizations and 18.19% and 13.24% of deaths in both MOH hospital and private hospital respectively.

Most ARTI cases in early childhood are confirmed as upper respiratory infection, leading to symptoms of the common cold with coryza and cough. In contrast, around one-third of infants with ARTI develop lower respiratory tract symptoms such as tachypnea, wheezing, severe cough, breathlessness and respiratory distress (Tregoning & Schwarze, 2010). Although lower respiratory tract infections (LRTI) are less frequent than upper respiratory tract infections (URTI), more patients with LRTI are hospitalized (Templeton, 2007).

In developing countries, although bacterial pathogens contribute to the cause of severe lower respiratory tract disease, most LRTI are indeed caused by respiratory viruses. In fact, sometimes respiratory infections that start out as a viral infection can lead to

secondary bacterial infections (Calvo *et al.*, 2008; Peltola & Ruuskanen, 2008). Antibiotics, which are often stereotyped as a cure-all for common colds and fevers, work only for bacterial, but not viral infections. Nonetheless, they are often inaccurately prescribed for viral illnesses. Current management of ARTI is suboptimal in most countries. The lack of knowledge about common viral diseases may lead to overuse of antibiotics, which in turn contributes to the emergence of antibiotic-resistance bacterial pathogens (Tregoning & Schwarze, 2010).

A lot of data has been published in the public domain regarding RSV, HRV, HPIV, IFV infections. Moreover, despite the common occurrence of ARTI globally, most of the supportive data available are from the temperate area. Data from the tropics and subtropics are very scant. There is also very little published data on epidemiology of viral infections in Borneo. In recent years, new human respiratory viruses have been reported including HMPV, HBoV, and four new human coronaviruses including Severe Acute Respiratory Syndrome coronavirus (SARS-CoV), HCoV-NL63, HCoV-HKU1 and MERS-CoV, thus increasing the urgency for the study of epidemiology of respiratory tract pathogen infections in developing countries.

Data on disease burden are unavailable from many countries, especially those that are under-resourced and, at present, there is no globally integrated surveillance system for respiratory viral infections (RVIs) other than influenza and RSV. Supporting data on the epidemiology of respiratory illness in Malaysia especially Sarawak is rather scarce. Therefore, understanding the geographical patterns, seasonality and distributions of RVIs would allow for more appropriate and targeted clinical management in situations where the burden of disease currently remains hidden. The discovery of antiviral medicines in the late 20th century led to significant breakthroughs in the fight against infectious diseases.

Furthermore, the wider scale use of effective vaccines for viral infections (e.g. influenza) and bacterial infections (e.g. Hib and pneumococcal conjugate) will probably lead to changes in the etiologies and, perhaps, patterns of serious respiratory illnesses. Besides that, further information is needed on the role of RVIs in causing the clinical syndromes, in order to improve strategies to reduce the overall morbidity and mortality of acute respiratory disease.

The clinical presentation might be similar for ALRI caused by different agents, but the epidemiological impact differs among several causative agents (Dut & Kocagöz, 2016). Different viruses have different patterns of seasonality, which make infection possible year round. The variation in incidence of respiratory viral infection is distinct in temperate regions. In tropical countries, correlation of respiratory viral activity with climatic factors is not so well defined (Baumgartner *et al.*, 2012).

There is contrast shown in the seasonality between the studies done in Malaysia (Khor *et al.*, 2012) and Singapore (Shek & Lee, 2003) although these are neighbouring countries. This shows that epidemiological differences might exist for ARTI between different regions due to differences in climatic change and humidity. In addition, the seasonality may vary according to latitude and climatic zone. Epidemiological studies from tropical regions indicate that factors such as rainy season or low air humidity in addition to temperature may influence respiratory viruses outbreaks (Chew *et al.*, 1998). Global warming carries profound changes in Earth's climate and consequently, climatic variations and extreme weather changes also cause profound impacts on infectious diseases incidences (Mirsaeidi *et al.*, 2016). As there is no epidemiological study in Borneo, seasonality of ARTI in Borneo remains unknown.

With the progress in molecular biology and availability of more sophisticated diagnostic tests, multiple respiratory viruses are now often detected in ARTI, especially in children. Such observations lead to understanding about disease causation, the dynamics of infection with multiple agents and others. In Malaysia, there is limited onsite diagnostic testing for viral diseases. Diagnoses are often made on clinical observations. If physicians are well-informed of the clinical presentation of common viral infections, coupled with information derived from on-going viral disease surveillance, the care of children with ARTI will be further improved. The causative pathogens and particularly the contributions of RVIs need to be better characterized in different clinical syndromes, settings and target populations. With the innovative approaches targeting broad spectrum of pathogen, it would be beneficial to consider innovative therapeutic approaches that do not focus on a single virus. In future, when these medications are combined with advances in diagnostics tests and improvements in clinical management, it seems that effective treatment of RVIs is a real possibility.

To succeed in the battle against respiratory viruses, we need to understand the burden of disease in the population. Only then can we assemble the findings for future needs of vaccines usage and therapeutics against respiratory viruses, and make them available to those in need. Thus, the present study using prospective data was carried out with the above in view.

1.9 General Objective

The primary objective of this study is to investigate the epidemiology of severe community-acquired viral lower respiratory tract infection in children aged 5 years and below in Sarawak.

1.10 Specific objectives

i. *To describe the epidemiology and seasonality of acute viral respiratory tract infections among young children aged five years and below.*

   To describe the clinical diagnosis, putative causative agents and seasonality of severe community-acquired lower respiratory tract infections in children aged 5 years and below in Sarawak.

ii. *To examine the molecular genetics characterization of main respiratory viruses among young children aged five years and below.*

   To study the phylogenetic profiles of the main respiratory pathogens detected in Sarawak compared to those identified elsewhere.

1.11 Research Hypotheses

i. The main viral cause of ARTI will be respiratory syncytial virus (RSV).
ii. Respiratory virus detection will be found in more than 50% of acute viral respiratory tract infections among young children aged five years and below.

iii. ALRI caused by viruses such as RSV and influenza virus will have a strong seasonal pattern.

iv. Respiratory viruses such as RSV, HAdV, HRV will show the presence of different viruses' subgroup and lineages.

CHAPTER 2

MATERIALS AND METHODS

2.1 Study design and location

An epidemiological study was conducted among children with ARTI aged five years and below who were admitted into the High Dependency Unit and Intensive Care Unit at 3 major hospitals in Sarawak. Participating hospitals included Sarawak General Hospital in Kuching (SGH) (A), Sibu Hospital (B) and Bintulu Hospital (C) as shown in Figure 2.1. All experimental work associated with this study was done at the Institute of Health and Community Medicine (IHCM), Universiti Malaysia Sarawak after obtaining approval from the Medical Research and Ethics committee (MREC) of the Ministry of Health Malaysia (NMRR-12-1298-13465) (Appendix A). Respiratory specimens were collected in children under five years over a 3-year period between November 2012 and October 2015. Informed consent was obtained from each parent or guardian prior to enrolment.



Figure 2.1: Locality map

2.2 Study population and subjects

Children aged five years or younger with lower respiratory tract manifestations of ARTI were eligible for inclusion. A child may be recruited into the study anytime between the time of hospital admission and first 48 hours after hospital admission. Acute lower respiratory tract infection was defined as per WHO Guidelines (WHO, 1991). A child must fulfil **ALL** the three criteria below before he/she was enrolled into the study:

1. Age between 1month and 5 years (60 months)

   PLUS

2. Lower respiratory tract infection (LRTI)
   - Defined by the presence of:

     a. History of fever at home or fever on examination (axillary temperature ≥37.5C)

     AND

     respiratory symptoms (such as cough, rhinorrhoea, stridor, wheezing, rapid breathing, dyspnoea)

     AND

     b. abnormalities

        i. on physical examination including age-specific tachypnoea [i.e. ≥50/min in children aged between 1 and 12 months; ≥40/min in children aged between >12 months and 5 years], respiratory distress, wheezing, crackles, bronchial breathing and O_2 saturation at room air ≤ 94%.

        OR

        ii. on chest radiograph such as air-trapping, infiltrates, air-bronchogram, pleural effusions.

PLUS

3. Signs of severe disease
   - Defined by any one of the followings:
     i. Chest in-drawing (inward movement of the bony structures of the lower chest wall with each inspiration i.e. "when the child breaths in, the lower chest goes in".)
     ii. Stridor in a calm child
     iii. Unable to drink or breastfeed normally
     iv. Repeated vomiting after drinking or breastfeeding
     v. Convulsion or history of convulsion before presentation
     vi. Altered mental state including lethargy, drowsiness (abnormally sleepy), inconsolable cry (irritability)

Exclusion criteria:

1. Children with respiratory symptoms such as cough and rhinorrhea **WITHOUT age-specific tachypnoea** are considered to have acute upper respiratory tract infection.
2. Children with LRTI **WITHOUT** signs of severe disease.
3. Respiratory symptoms that occurs after 48 hours of admission is considered nosocomial in origin and thus not eligible for inclusion to the study.

2.3 Respiratory samples

2.3.1 Types of sample

Respiratory specimens collected in this study included nasopharyngeal aspirates (NPA), nasopharyngeal swabs (NS) and/or endotracheal tube secretions (ETT), if the patient was intubated. These samples were also the common sample types routinely collected in day to day clinical practice for such cases. All samples were collected using standard hospital protocols.

2.3.2 Collection protocol

a) Nasopharyngeal aspirate (NPA)

i. Nasopharyngeal secretions were aspirated through a catheter connected to a mucus trap and fitted to a vacuum source.

ii. The catheter was inserted into the nostril parallel to the palate. The vacuum was applied and the catheter was slowly withdrawn with a rotating motion.

iii. Mucus from the other nostril was collected with the same catheter in a similar manner.

iv. After mucus had been collected from both nostrils, the catheter was flushed with 3 ml of viral transport medium (VTM).

b) Nasopharyngeal swab (NS)

i. A flexible, nasopharyngeal flocked swab was inserted through the nostril and to the nasopharynx and left in place for a few seconds.

ii. It was then slowly withdrawn with a rotating motion.

iii. The tip of the swab was put into a vial containing 2-3 ml of virus transport medium and the shaft cut.

iv. The procedure was repeated on the other nostril with a new swab.

v. Both swabs were put into a single VTM.

c) Endotracheal Tube (ETT)

i. Collection of endotracheal tube secretion was done by using a catheter connected to a mucus tray and fitted into a vacuum source.

ii. After ETT mucus had been collected from both nostrils, the catheter was flushed with 3 ml of VTM.

2.3.3 Sample processing

Specimens collected were frozen at -80 ^{0}C upon collection. All samples were transported on ice to IHCM for further investigation. Samples obtained were cultured on 4 different cell lines (MDCK, A549, Vero, RD) for further analysis if needed. Cultures were then examined daily for cytopathic effect (CPE). Blind passage was attempted if initial inoculation did not show a CPE effect. Cultures with CPE were harvested and further examined by PCR. Simultaneously, primary samples were aliquoted for nucleic acid extraction and PCR investigation using published and in-house protocols for primary screening of respiratory viruses. Figure 2.2 shows a summarized flow chart of this process.

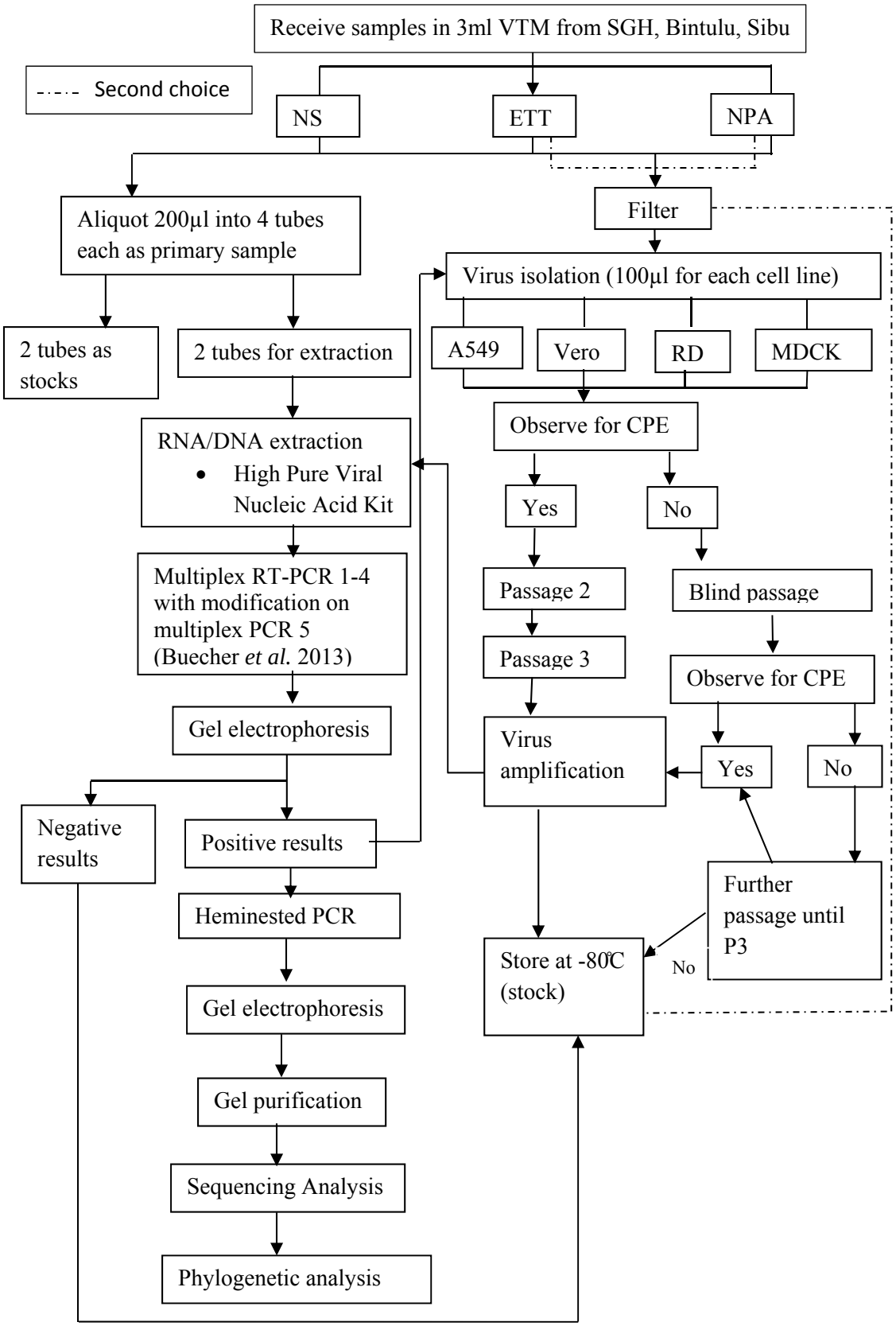


Figure 2.2: Flow chart of methodology

2.4 Cell culture and virus isolation

2.4.1 Media and solutions

Table 2.1: Preparation for media and solutions

Cell culture media	Preparation
10% complete RPMI 1640 (Gibco BRL, Life Technololgies, Rockville, MD, USA) growth culture medium	10ml of 10% foetal bovine serum (FBS) + 1ml Penicillin G, final conc. 100 U/ml + 1ml Streptomycin sulphate, final concentration, 100 µg/ml, Sigma, MO, USA) in 100ml RPMI 1640 medium
5% RPMI (Gibco BRL, Life Technologies, Rockville, MD, USA) maintenance medium	5ml of 5% foetal bovine serum (FBS) + 1ml Penicillin G, final conc. 100 U/ml + 1ml Streptomycin sulphate, final concentration, 100 µg/ml, Sigma, MO, USA) in 100ml RPMI 1640 medium
10% complete DMEM (Gibco BRL, Life Technologies, Rockville, MD, USA) growth culture medium	10ml of 10% foetal bovine serum (FBS) + 1ml Penicillin G, final conc. 100 U/ml + 1ml Streptomycin sulphate, final concentration, 100 µg/ml, Sigma, MO, USA) in 100ml DMEM medium
5% DMEM (Gibco BRL, Life Technologies, Rockville, MD, USA) maintenance medium	5ml of 5% foetal bovine serum (FBS) + 1ml Penicillin G, final conc. 100 U/ml + 1ml Streptomycin sulphate, final concentration, 100 µg/ml, Sigma, MO, USA) in 100ml DMEM medium
5% FBS Opti-MEM (Gibco BRL, Life Technologies, Rockville, MD, USA) (growth media)	5ml of 5% foetal bovine serum (FBS) + 1ml Penicillin G, final conc. 100 U/ml + 1ml Streptomycin sulphate, final concentration, 100 µg/ml, Sigma, MO, USA) in 100ml Opti-MEM medium
0.2% BSA Opti-MEM (Gibco BRL, Life Technologies, Rockville, MD, USA) (maintenance medium)	4ml of 5% foetal bovine serum (FBS) + 1ml Penicillin G, final conc. 100 U/ml + 1ml Streptomycin sulphate, final concentration, 100 µg/ml, Sigma, MO, USA) in 100ml Opti-MEM medium
Media for MDCK virus growth	10µl 2mg/ml TPCK-trypsin + 10ml 0.2% BSA Opti-MEM
Solution	**Preparation**
Phosphate buffered saline (PBS)	1.15g Na2PO4 (anhydrous) +0.2g KH2PO4 + 89g NaCl added to be 1L
PBS-EDTA	0.465gEDTA diNa salt in 500ml PBS
TPCK-trypsin stock solution	20mg TPCK-trypsin in 10ml of PBS-EDTA (final concentration 2mg/ml)
1% Trypsin (Sigma-Aldrich, St. Louis, MO, USA)	1g 1:250 Trypsin powder in 100 ml sterilized PBS
0.1% Trypsin-EDTA	10ml of 1% Trypsin + 90ml PBS-EDTA

2.4.2 HRV and HAdV cell culture condition and virus isolation

For HRV, RD (*rhabdomyosarcoma)* cell line was grown using RPMI growth medium (Table 2.1) at 33^0C. As for HAdV, A549 cell line was grown using DMEM growth medium at 37^0C. Culturing of the cell monolayer was performed in 75 cm tissue culture flasks. The growth medium was discarded and replaced with 5% RPMI maintenance medium for HRV and 5% DMEM maintenance medium for HAdV (Table 2.1) after two days. Confluent cells (between 80-100%) were washed with PBS, detached using 3 ml of 0.1% Trypsin-EDTA and re-suspended with new growth medium. The cell suspension was split according to the propagation factor of 1:4 into new flasks for virus isolation.

Virus isolation were done on 24-well plates (Corning Costar Corporation, NY USA). 0.5 ml/well of RD / A549 cells suspension (2-3 x 10^5 cells/ml) in 5% RPMI medium and 5% DMEM medium were dispended throughout the 24 wells. 10 µl of filtered positive samples (as determined by RT-PCR) were inoculated into the well. The plate was then incubated at 33^0C and 37^0C in the presence of 5% CO_2 for HRV and HAdV culture respectively. Cell culture were feed with another 0.5ml/well of 5% FBS RPMI for RD cells 1 day after inoculation and 4-5 days after inoculation to avoid having acidic cultures. The cell culture plate was examined daily for virus growth by a cytopathic effect (CPE). Following 14 days of culture, any cultures which did not show CPE were subjected to a blind passage (passage 2) for another 14 days. Upon observation of more than 80% CPE, the contents of the well were harvested and stored at -80^0C. A second passage also be done on CPE positive cultures to ensure that the effect seen is not merely due to cytotoxic effects of the inoculum.

2.4.3 Influenza virus cell culture condition and virus isolation

For influenza virus, MDCK cell line was grown using 5% FBS Opti-MEM growth medium (Table 2.1) at 37^0C. Culturing of the cell monolayer was performed in 75 cm tissue culture flasks. The growth medium was discarded and replaced after two days. Confluent cells (between 80-100%) were washed with 5 ml PBS, detached using 2 ml of 0.1% TPCK-trypsin and distributed it over entire cell sheet by gently rocking the flask for 1 min. TPCK-trypsin was removed with pipette. Another 1ml of TPCK-trypsin solution was added and distributed over entire cell sheet and the flask was incubated at 37^0C until all cells were detached from plastic surface (5-10 min). The flasks were shake and tapped to detach cells. 2 ml of 5% FBS Opti-MEM was added to inactivate the remaining trypsin and 1 ml of solution was discarded. Another 9 ml of 5% FBS Opti-MEM was added followed by pipetting up and down gently to break up cell clumps. 5 ml of the cell suspension was transferred to a new T-25 flasks. The remaining cell suspension were used to seed plate for cell passage. Finally, the flasks were incubated at 37^0C.

Virus isolation were done on 24-well plates (Corning Costar Corporation, NY USA). 0.5 ml/well of MDCK cells suspension (2-3 x 10^5 cells/ml) in 5% FBS Opti-MEM medium were dispended throughout the 24 wells. The plate was then kept in a plastic container and incubated overnight at 37^0C in the presence of 5% CO_2. Media was then discarded and washed twice with 1 ml of PBS. 0.5 ml of 0.2% FBS Opti-MEM treated with 2µg/ml of TPCK-Trypsin was added into each well. 10 µl of filtered positive samples (as determined by RT-PCR) were inoculated into the well. One or two wells per plate were left uninoculated to serve as controls. The plate was then incubated at 37^0C in the presence of 5% CO_2. Cell culture were feed with another 0.5ml/well of 0.2% FBS Opti-

MEM treated with 2µg/ml of TPCK-Trypsin 1 day after inoculation and 4-5 days after inoculation to avoid having acidic cultures. The cell culture plate was examined daily for virus growth by a cytopathic effect (CPE). Following 14 days of culture, any cultures which did not show CPE were subjected to a blind passage (passage 2) for another 14 days. Upon observation of more than 80% CPE, the contents of the well were harvested and stored at -80^{0}C. A second passage also be done on CPE positive cultures to ensure that the effect seen is not merely due to cytotoxic effects of the inoculum.

2.5 Molecular diagnostics methods

2.5.1 Nucleic acid extraction

The extraction of viral nucleic acid was done using the High Pure Extraction Kit (Roche Diagnostics GmbH, Mannheim, Germany) which enables the extraction of both RNA and DNA through a single extraction. The extraction was carried out following instructions provided by the manufacturer with slight modifications.

Generally, for a single extraction procedure, 200 µl of binding buffer [6 M guanidine-HCL, 10 mM Tris-HCL, 20% Triton® X-100 (w/v), pH 4.4 (25°C)] was combined with 200 µl of sample in a 1.5 ml Eppendorf tube. A volume of 55 µl of a premixed working solution containing 50 µl of 18 mg/ml proteinase K and 5 µl of 4 mg/ml poly (A) carrier RNA was added before the contents were mixed by inverting the tube several times and incubated at 72°C in a water bath for 10 minutes to lyse the virus. After incubation, 100 µl of isopropanol was added into the mixture and gently mixed by

inverting the tube several times. The sample was then pipetted onto the capture column pre-assembled in a disposable collection tube provided by the manufacturer.

The contents were centrifuged at 13,400 rpm for 1 minute in a Minispin® bench-top mini centrifuge (Eppendorf, Hamburg, Germany), in which the extracted viral nucleic acids were bound to the glass fiber fleece on the filter of the capture column. The flow-through was discarded together with the collection tube and the capture column transferred to a fresh collection tube. 500 µl of inhibitor removal buffer to the capture column and centrifuged at 13,400 rpm for 1 minute. The flow-through was discarded together with the collection tube and capture column transferred to a fresh collection tube followed by two centrifugation (13,400 rpm for 1 minute) wash steps with 450 µl of wash buffer [20 mM NaCl, 2 mM Tris-HCL, pH 7.5 (25°C) final concentrations after addition of ethanol] to remove enzymes, salts and impurities. After the final wash, the flow-through was discarded and the assembly centrifuged at 13,400 rpm for an additional 1 minute to remove residual wash buffer from the capture column. The capture column was dried in the oven at 80°C for 5 minutes to completely remove all residual alcohol from the wash steps. Nucleic acids bound to the filter was eluted by adding 35 µl elution buffer [nuclease-free, sterile, double distilled water (ddH_2O)] provided with the kit and centrifuged for 2 minutes in a bench-top mini centrifuge model 5415C (Eppendorf, Hamburg, Germany) and stored at -80°C until used.

2.5.2 Multiplex RT-PCR

In this study, a previously published multiplex PCR/RT-PCR assay adapted from Buecher *et al.*, (2010) targeting 18 respiratory viruses was used. In this assay, viruses were

detected using five separate multiplex PCR reactions performed separately (RT-PCR 1 to 4 and PCR 5). IAV, IBV, HMPV and RSV (A and B) were detected by the multiplex RT-PCR 1; HPIV-1, -2, -3, and -4 by the multiplex RT-PCR 2; HRV, enterovirus (EV), ICV and SARS-CoV by the multiplex RT-PCR 3; HCoV-OC43, -229E, -NL63 and –HKU1 by the multiplex RT-PCR 4; HAdV and HBoV by multiplex PCR 5 with slight modifications (Table 2.1).

Multiplex RT-PCR 1, 2, 3 and 4 are a single-step combined PCR amplification performed using the SuperScript® III One-Step RT-PCR with Platinum® *Taq DNAPolymerase* (Invitrogen/Life Sciences). The four assays were undertaken in a 30µl reaction volume containing 5µl RNA extract, 1µl of premix primer (10µM), 1 µl SuperScript®III RT/ Platinum®*Taq*Mix, 15 µl of 2X Reaction Mix (a buffer containing 0.4 mM of each dNTP, 3.2 mM $MgSO_4$) and RNase-free water to complete the 30µl final volume. The reaction was carried out in a Eppendorf Master Cycler (Eppendorf, Hamburg, Germany) with an initial reverse transcription step at 50˚C for 1 hour, followed by PCR activation at 94˚C for 2 min, 8 initial cycles of amplification (30 sec at 94˚C, 30 sec at 58˚C, 1 min at 72˚C), 30 additional cycles of amplification (30 sec at 94˚C, 30 sec at 50˚C, 1 min at 72˚C) and a final extension step at 72˚C for 10 min. Multiplex RT-PCR products were visualized after gel electrophoresis on an ethidium bromide-stained 2% agarose gel.

2.5.3 Hemi-nested PCR

The PCR product yields from primary samples were generally low for confirmation by sequencing. Thus, the products of multiplex RT-PCRs 1, 2, 3 and 4 were subjected to hemi-nested PCR to increase detection yields if positive results were obtained from the

screening of multiplex RT-PCR. The principle is to amplify part of one or several DNA fragments resulting from RT-PCR. For each virus, an internal primer was used together with the corresponding either one of the primer used for RT-PCR (Table 3.1).

For the hemi-nested PCR, 1:100 dilution or 5µl of neat products of multiplex RT-PCR were used as templates for amplification in single PCR carried out in a final volume of 20µl. A master mix was prepared containing 10X *Taq* buffer with $(NH_4)_2SO_4$ [containing 750 mM Tris-HCl (pH 8.8 at 25ºC), 200 mM $(NH_4)_2SO_4$, 0.1% Tween 20], 1.5 mM $MgCl_2$, 2.5 units of recombinant *Taq* DNA polymerase (MBI Fermentas, Vilnius, Lithuania), 10 mM dNTP Mix (MBI Fermentas, Vilnius, Lithuania), sterile UHQ water and 10 pmol/µl of the anti-sense and sense primers. We added 5 µl of each multiplex RT-PCR product to this mixture. For every hemi-nested PCR run, water control was included.

The PCR cycling conditions were as follows: initial denaturation at 94ºC for 2 minutes, 40 cycles of denaturation at 94ºC for 30 seconds, primer annealing at 58ºC for 30 seconds and extension at 72ºC for 1 min; and a final extension at 72ºC for 10 minutes.

2.5.4 Gel electrophoresis

The PCR product was analyzed by gel electrophoresis in a 2% (w/v) molecular biology-grade agarose gel (Amresco, Solon, OH, USA) at 150V using EasyCastTM Mini Electrophoresis System (Owl, Portsmouth, NH, USA). All gels contained 0.5 ug/ethidium bromide as staining agent in 1x Tris-Borate EDTA (TBE) buffer (BioSyntech, Selangor, Malaysia). The gel was viewed under Gel Doc^{TM} XR^{+} Gel Documentation System (BioRad, California, USA) to check for the expected size of PCR product as described in

Table 2.1. The GeneRuler^{TM} 100bp DNA ladder (MBI Fermentas Vilnius, Lithuania) was used as a reference maker.

2.5.5 Purification of DNA fragments from agarose gel

All purification of DNA fragments in this study were done using the QIAquick Gel Extraction Kit (QIAGEN, 2008). This protocol is designed to extract and purify DNA of 70bp to 10kb from standard or low-melt agarose gels in TAE or TBE buffer. After analyzing the PCR product by gel electrophoresis, the expected DNA fragments were excised from the gel using a sterile disposable blade. Three volumes of Buffer QG (5.5M guanidine thiocyanate; 20mM Tris-HCl pH 6.6) were added to 1 volume of excised gel. The Buffer QG gel mixture was then incubated at 50˚C for about 10 minutes until the gels were completely dissolved. Every three minutes, the tube was vortexed to ease the dissolving of gel in the buffer. Following this, 100ml of isopropanol was added to increase the yield of product which is less than 500bp. The mixture was then transferred into a QIAquick spin column and centrifuged for one minute to bind the DNA to the membrane. The flow-through was discarded and 500ml of Buffer QG was once again added to the spin column and centrifuged for one minute to ensure complete removal of agarose gel traces in the column or form the DNA. Buffer PE (10mM Tris-HCl pH 7.5; 80% ethanol) was added for washing step. The spin column was left to stand at room temperature for 2 minutes before centrifugation for one minute. After the flow-through was discarded, additional centrifugation for one minute is necessary to ensure residual ethanol was completely removed. The residual ethanol was dried from the spin column in the oven at 70˚C for 5 minutes. Sterile UHQ water was used in the elution step in which it was added

to the center of the spin column membrane, incubated at room temperature for one to two minutes and centrifuged for 2 minutes.

All centrifugation done in this method was set at 12,000 x g on the benchtop minicentrifuge, the Minispin®,(Eppendorf, Hamburg, Germany). As the purified DNA will be used as template in sequencing, it is crucial to completely remove all traces of agarose and ethanol as these elements will interfere with the sequencing process and generate bad sequence read.

Table 2.2: Primers and sequence information

Virus	Assay	Primer designation	Primer sequences (5'-3')	Gene	size (bp)	Refs.
Multiplex 1						
IAV	RT-PCR	M1_IAV_F	CAG AGA CTT GAA GAT GTC TTT GCT AG	Matrix Protein	212	Donofrio *et al.* 1992
		M1_IAV_R	GCT CTG TCC ATG TTA TTT GGA ATC			
	Heminested	M1_IAV_hF	CTC TGA CTA AGG GGA TTT TG		130	Bellau-Pujol *et al.* 2005
IBV	RT-PCR	M1_IBV_F	GAA AAA TTA CAC TGT TGG TTC GGT G	Matrix Protein	365	Donofrio *et al.* 1992
		M1_IBV_R	AGC GTT CCT AGT TTT ACT TGC ATT GA			
	Heminested	M1_IBV_hR	CAT GAA ARC TCA CAC ATC T		260	Bellau-Pujol *et al.* 2005
RSV	RT-PCR	M1_RSV_F	GGA ACA AGT TGT TGA GGT TTA TGA ATA TGC	Nucleocapsid	278	Cane and Pringle 1991
		M1_RSV_R	CTG CTG TCA AGT CTA GTA CAC TGT AGT			
	Heminested	M1_RSV_hR	GGT GTA CCT CTG TAC TCT C		180	Bellau-Pujol *et al.* 2005
HMPV	RT-PCR	M1_HMPV _F	GGA GTC CTA CCT AGT AGA C	Matrix Protein	537	Buecher. *et al.* 2010
		M1_HMPV _R	GCA GCT TCA ACA GTA GCT G			
	Heminested	M1_HMPV _hF	AGG CCA ACA CAC CAC CAG		410	Bellau-Pujol *et al.* 2005
Multiplex 2						
HPIV-1	RT-PCR	M2_HPIV 1_F	CCG GTA ATT TCT CAT ACC TAT G	Hemagglutinin-Neuraminidase	317	Echevarria *et al.* 1998
		M2_HPIV 1_R	CTT TGG AGC GGA GTT GTT AAG			
	Heminested	M2_HPIV 1_hF	AGC TGC AGG AAC AAG GGG		261	Bellau-Pujol *et al.* 2005
HPIV-2	RT-PCR	M2_HPIV 2_F	AAC AAT CTG CTG CAG CAT TT	Hemagglutinin-Neuraminidase	507	Echevarria *et al.* 1998
		M2_HPIV 2_R	ATG TCA GAC AAT GGG CAA AT			
	Heminested	M2_HPIV 2_hF	CTA GCT GAA CTG AGA CTT G		340	Bellau-Pujol *et al.* 2005
HPIV-3	RT-PCR	M2_HPIV 3_F	CTC GAG GTT GTC AGG ATA TAG	Hemagglutinin-Neuraminidase	189	Karron *et al.* 1994
		M2_HPIV 3_R	CTT TGG GAG TTG AAC ACA GTT			
	Heminested	M2_HPIV 3_hR	GCT AGA GAA CAT GAC TTC C		145	Bellau-Pujol *et al.* 2005
HPIV-4	RT-PCR	M2_HPIV 4_F	CTG AAC GGT TGC ATT CAG GT	phosphoprotein	451	Aguilar *et al.* 2000
		M2_HPIV 4_R	TTG CAT CAA GAA TGA GTC CT			
	Heminested	M2_HPIV 4_hR	GTC TGA TCC CAT AAG CAG C		390	Bellau-Pujol *et al.* 2005

Table 2.2 continued

Multiplex 3						
ICV	RT-PCR	M3_ICV_F	ACA CTT CCA ACC CAA TTT GG	Hemagglutinin-Esterase	485	Zhang and Evans 1991
		M3_ICV_R	CCT GAC AGC AAC TCC C TC AT			
	Heminested	M3_ICV_hF	GAG GAT GTG GCA ACT ACT		391	Bellau-Pujol *et al.* 2005
HRV/EV	RT-PCR	M3_HRhV_F	GCA TCI GGY ARY TTC CAC CAC CAN CC	VP4/VP2/5'NC	549	Savolainen *et al.* 2002
		M3_HRhV_R	GGG ACC AAC TAC TTT GGG TGT CCG TGT			
	Heminested	M3_HRhV_hR	ATG GGN GCW CAN GTN TCH ANH CA		450	Bellau-Pujol *et al.* 2005
SARS-CoV	RT-PCR	M3_SARS_COV_F	CAT AAC CAG TCG GTA CAG CTA	Gene M	195	Drosten *et al.* 2003
		M3_SARS_COV_R	RTG AAT TAC CAA GTC AAT GGT			
	Nested	M3_SARS_COV_NF	CTG TAG AAA ATC CTA GCT GGA		110	Drosten *et al.* 2003
		M3_SARS_COV_NR	GAA GCT ATT CGT CAC GTT CG			
Multiplex 4						
HCoV-OC43	RT-PCR	M4_HCoV-OC43_F	GGC TTA TGT GGC CCC TTA CT	Gene M	334	Vabret *et al.* 2001
		M4_HCoV-OC43_R	GGC AAA TCT GCC CAA GAA TA			
	Heminested	M4_HCoV-OC43_hR	CTC CAA AAA CTT CCA GTT C		170	Bellau-Pujol *et al.* 2005
HCoV-229E	RT-PCR	M4_HCoV_229E_F	TGG CCC CAT TAA AAA TGT GT	Gene M	574	Vabret *et al.* 2001
		M4_HCoV-229E_R	CCT GAA CAC CTG AAG CCA AT			
	Heminested	M4_HCoV-229E_hR	CCG TAT CAA CAC TCG TTA TGT GG		230	Bellau-Pujol *et al.* 2005
HCoV-HKU1	RT-PCR	M4_HCoV-HKU1_F	ATC TGA GCG AAA TTA CCA AAC	Gene M	443	Vabret *et al.* 2005
		M4_HCoV-HKU1_R	CGG AAA CCT AGT AGG GAT AGC TT			
HCoV-NL63	RT-PCR	M4_HCoV-NL63_F	GAT AAC CAG TGG AAG TCA CCT AGT TC	Gene M	255	Vabret *et al.* 2005
		M4_HCoV-NL63_R	ATT AGG AAT CAA TTC AGC AAG CTG TG			
Multiplex 5						
HAdV	PCR	M5_HAdV_HEX B1	AAC ATG ACC AAR GAC TGG TT	Hexon gene	540	Cardosa *et al.*, 1999
		M5_HAdV_HEX B2	GCC GAG AAS GGY GTR CGC AGG TA			
	Nested PCR	M5_HAdV_HEX B3	TTC AGA AAC TTC CAG CCY ATG AG		350	Cardosa *et al.*, 1999
		M5_HAdV_HEX B4	TCC ATG GGA TCC ACC TCA AAR GTC AT			
HBoV	PCR	HBoV_F	GAS CTC TGT AAG TAC TAT TAC	NP-1 gene	354	Allander *et al.* 2005
		HBoV_R	CTC TGT GTT GAC TGA ATA CAG			

2.5.6 Optimization of Multiplex PCR assay for the detection of Human adenovirus and Human bocavirus

The original multiplex assay (PCR 5) described by Buecher and colleagues for the detection of HAdV and HBoV was suspected to be under-detecting HAdV infections based on the clinical descriptions of the relevant cases. To overcome this problem, a previously described nested PCR for HAdV (Cardosa *et al.*, 1999) was used in the multiplex PCR to replace the original HAdV primers described by Buecher and colleagues. In this modified multiplex protocol, the initial outer primer set of the Cardosa *et al.* (1999) HAdV primers were used in a multiplex together with the HBoV primers. HAdV detection was further achieved separately by a second nested PCR using the inner primer set of the HAdV primers of Cardosa *et al.* (1999). Primer sequences for HAdV detection are given in Table 2.2.

Briefly, in the modified PCR 5 protocol, nucleic acids from respiratory samples were extracted using High Pure Extraction Kit (Roche Diagnostics GmbH, Mannheim, Germany) according to manufacturer's instructions. PCR amplification was performed using 20µl reaction volumes containing 15µl of PCR reaction mixture which included 10X *Taq* buffer with $(NH_4)_2SO_4$ [containing 750 mM Tris-HCl (pH 8.8 at 25ºC), 200 mM $(NH_4)_2SO_4$, 0.1% Tween 20], 1.5 mM $MgCl_2$, 2.5 units of recombinant *Taq* DNA polymerase (MBI Fermentas, Vilnius, Lithuania), 10 mM dNTP Mix (MBI Fermentas, Vilnius, Lithuania), 10 pmol/µl of each primer, sterile UHQ water and 5µl of nucleic acid extract. PCR cycling conditions included initial denaturation at 94ºC for 2 minutes, 40 cycles of denaturation at 94ºC for 30 seconds, primer annealing at 58ºC for 30 seconds and extension at 72ºC for 1 min; and a final extension at 72ºC for 10 minutes. In the nested HAdV PCR reaction, 5 µl of a1:100 dilution of the first-round PCR reaction was amplified using specific primers

(Table 2.2) and reaction conditions similar to the first-round PCR as described above. PCR products were separated by electrophoresis on2% agarose containing 1µg/ml of ethidium bromide for 40 minutes at 170 V. Amplified products were visualized and documented using the Gel Doc XR+ System (Bio-Rad, California, USA).

Table 2.3: Primers sequences for Multiplex 5 amplification

Primer designation	**Primer sequences (5'-3')**	**Gene**	**Amplicon size (bp)**
Multiplex 5 (1st PCR)			
M5_HAdV_HEX B1	AAC ATG ACC AAR GAC TGG TT	Hexon gene	562
M5_HAdV_HEX B2	GCC GAG AAS GGY GTR CGC AGG TA		
HBoV_F	GAS CTC TGT AAG TAC TAT TAC	NP-1 gene	354
HBoV_R	CTC TGT GTT GAC TGA ATA CAG		
Nested PCR			
M5_HAdV_HEX B3	TTC AGA AAC TTC CAG CCY ATG AG	Hexon gene	349
M5_HAdV_HEX B4	TCC ATG GGA TCC ACC TCA AAR GTC AT		

2.5.7 Human rhinovirus phylogenetic analysis for VP4/VP2 region

HRV/EV positive samples from hospitalized patients were further identified by RT-PCR through amplification of the complete capsid protein VP4 and portions of the 5' end of the VP2 gene. RNA was first reverse transcribed into cDNA by using a reverse transcription step performed in a 10 µl reaction volume using the extracted RNA as template. 5µl of extracted viral nucleic acids was incubated at 70ºC for 10 minutes with 1 µl (10 pmol/µl) of outer anti sense primer: HRV OAS (5' ACATRTTYTSNCCAAANAYDCCCAT-3') as described in a previous study (Wisdom *et al.*, 2009)to denature RNA secondary structures. During the incubation, a master mix containing 5X RT reaction buffer [250 mM Tris-HCl (pH 8.3 at 25ºC), 250 mM KCl, 20 mM $MgCl_2$, and 50 mM DTT], 10 mM dNTPs (MBI Fermentas, Vilnius, Lithuania) and 100 units of Moloney Murine Leukimia Virus (M-MuLV) Reverse Transcriptase (MBI Fermentas, Vilnus, Lithuania) was prepared.

After 10 minutes of incubation, the reaction mixture was immediately kept on ice for 2 minutes to prevent the reformation of secondary structures and to allow the anti-sense primer to anneal to the complementary site. A total of 4 µl of master mix was then added to the RT-reaction mixture and incubated at 37°C for 1 hour followed by enzyme inactivation at 70°C for 10 minutes. 5µl of the cDNAs was used as templates for amplification by PCR in a 50 µl reaction volume. A master mix was prepared containing 10X *Taq* Buffer with $(NH_4)_2SO_4$ [containing 750 mM Tris-HCl (pH 8.8 at 25°C), 200 mM $(NH_4)_2SO_4$, 0.1% Tween 20], 1.5 mM $MgCl_2$, 2.5 units of recombinant Taq DNA polymerase (MBI Fermentas, Vilnius, Lithuania), 10 mM dNTP Mix (MBI Fermentas, Vilnius, Lithuania), sterile UHQ water, 10 pmol/µl of the outer anti-sense and sense primers: HRV OS (5'-CCGGCCCCTGAATGYGGCTAA-3) and HRV OAS (5'-ACATRTTYTSNCCAAANAYDCCCAT-3') (Wisdom *et al.*, 2009). For every RT-PCR run, a positive control using isolate MR690/1 (confirmed HRV isolate) and water control were included.

The PCR cycling conditions were as follows: initial denaturation at 94°C for 5 minutes, 35 cycles of denaturation at 94°C for 20 seconds, primer annealing at 54°C for 30 seconds and extension at 72°C for 60 seconds; with a final extension at 72°C for 5 minutes.

Another nested PCR using the same PCR cycling conditions as described above was carried out using 1 µl of the first PCR product with the following primers: M13F_primer47_HRV_IF: (pos: 570-591nt)5'- CAG GAA ACA GCT ATG ACC TAC TTT GGG TGT CCG TGT TTC-3'; M13R_P1.204_HRV_IR: (pos: 1049-1069)5'-GTA AAA CGA CCA GAT CHG GHA RYT TCC AMC ACC A-3'(modified Tapparel *et al.*, 2011). The amplification products were visualized by gel electrophoresis in a 2% (w/v) molecular biology-grade agarose (Amresco, Solon, OH, USA) at 180V using EasyCast™

Mini Electrophoresis System (Owl, Portsmouth, NH, USA). The expected size of the PCR product was 499bp.

2.6 Sequencing and analysis

All positive results detected by heminested PCR will further be confirmed and subtyped by DNA sequencing. DNA sequencing in this study was carried out using an automated DNA Sequencer, 3130 Genetic Analyzer (Applied Biosystems Foster City, California, USA). Before commencing the cycle sequencing, sequencing PCR and the clean-up of the sequencing PCR products were performed.

2.6.1 Sequencing PCR

Prior the sequencing PCR, purification of the extracted DNA fragments from the agarose gel was determined using NanoDrop Spectrophotometer® ND-1000 v3.5 (NanoDrop Technologies, Inc., USA). The purity was measured in the form of 260/280 ratio. This ratio is a ratio of sample absorbance at 260 and 280 nm and used to access the purity of DNA and RNA. A ratio between 1.8 to 2.0 is generally accepted as 'pure' for DNA and if the ratio is appreciably lower or higher, it may indicate the presence of protein, phenol, salt or other contaminants that absorb strongly at or near 280 nm. Sequencing was performed in a 20 µl reaction volume. Briefly, the reaction included 4.5 µl of the premixed reaction from BigDye® Terminator v3.2 Cycle Sequencing Kit (Applied Biosystems, Foster City, California, USA)., 1 µl of 3.2 pmol/µl sequencing primer, 100-150 ng of PCR template and sterile UHQ water added to a final volume of 20 µl. The cycling conditions involved

initial denaturation at 94ºC for 5 minutes followed by 40 cycles of denaturation at 94ºC for 30 seconds; annealing at 50ºC for 30 seconds; and extension at 60ºC for 2 minutes.

2.6.2 Sequencing PCR clean up

The sequencing PCR products clean-up was carried out using BigDye® Sequencing Clean up Kit (McLab) and DynaMag™-96 Side Magnet (Thermo Fisher Inc., 2011). The kit consisted of BigDye®Cleaning Bead (BCB) and an elution buffer. Each component has been optimized for removing salts and unincorporated dye terminators from DNA sequencing reaction mixtures. DynaMag™-96 Side Magnet has been optimized to pull all types of Dynabeads® (range 1 – 4.5 µm in diameter to the sides of PCR tubes or to the sides of wells in 96-well plates.

The protocol used in this study is for a 96-well plate format. After a brief vortex of the BCB (to resuspend the beads), 20 µl of the BCB was added to 20 µl of sequencing PCR products and mixed evenly by pipetting. It was then left at room temperature for 5 minutes. 84 µl of 85% undenatured absolute ethanol was then added to the mixture and mixed gently. Following this, the 96-well plate was placed onto the 96-well magnetic plate (DynaMag™-96 Side Magnet) for 5 minutes until the solution was clear. While the plate was on the magnet, the supernatant was aspirated out from the wells and discarded. In the washing step, 100 µl of 85% ethanol was added to each well and incubated for 3 minutes at room temperature. After incubation, supernatant was aspirated out and discarded. The washing step was repeated twice and the plate remained on the magnetic throughout the process. The removal of supernatant was done thoroughly to avoid ethanol and other contaminants residues. To ensure complete removal of ethanol, the samples were dried in

the oven at 70ºC for 5 minutes. The plate was taken off the magnetic plate during the elution step and 15 µl of elution buffer was added onto each sample. The mixture was thoroughly mixed by pipetting until beads are fully resuspended and was then incubated at room temperature for 5 minutes. After the incubation, the plate was placed on magnetic plate and left to stand at room temperature for another 5 minutes until the solution was clear. For sequencing, 10 µl of cleared solution was transferred onto a new plate, cautiously avoiding beads transfer. The solution was then subjected to incubation at 94ºC for 2 minutes, to denature secondary structures prior to analysis by the Genetic Analyzer.

2.7 Analysis of sequenced data

Data and result obtained in this study were analyzed using LaserGene software (DNAStar Inc., Madisaon, WI, USA). The DNA sequences were compared to sequences deposited in the public database GenBank at the NCBI website using basic local alignment search tool (BLAST) (http://www.ncbi.nlm.nih.gov/BLAST/).

According to the results of blastn, if the nucleotide sequence identity of the PCR product of a samples is ≥ 90% to the prototype strain of a certain species, it will be assigned to this species; while if the nucleotide sequence identity of PCR product is less than 90% the virus strain will be considered untyped.

As for HRV/EV phylogenetic analysis, the HRV/EV in each sample was determined on the basis of the phylogenetic tree and by comparing it with the available HRV references encoding VP4/VP2 gene or full genome retrieved from Genbank (http://www.ncbi.nlm.nih.gov).Phylogenetic trees were constructed using MEGA 5.2 (Molecular Evolutionary Genetic Analysis) software package by the neighbour-joining

method applying the Kimura's two-parameter method using 1000 bootstrap replicates. All phylogenetic analysis was undertaken using the Enterovirus D sequence (GenBank accession no. D00820 for EV-D70) as an outgroup.

2.8 Data management and data analysis

Written informed consent (refer Appendix B) were obtained from the parents before samples were taken from the children. All experimental work associated with this study was done at the Institute of Health and Community Medicine (IHCM), Universiti Malaysia Sarawak after obtaining approval from the Medical Research and Ethics committee (MREC) of the Ministry of Health Malaysia. The pediatrician in-charge in the three respective hospitals were responsible to fill in the Study Form (refer Appendix C) and patient data collection form (refer Appendix D).

Samples were assigned sequential numbers. Patient's names or initials would not be used in any publications resulting from this study. Demographic, clinical, and virologic data were collected as well. These include patient age, gender, and ethnicity, underlying medical conditions, symptoms on admission, physical exam findings, oxygen requirement, intensive care unit admission, and length of hospitalization.

An electronic database which was only accessible to the investigators in this study was set up and stored in a UNIMAS archive indefinitely. A secure database, which was password protected and only be accessible to the investigators in this study was established. The database was also electronically backed up to prevent possible loss. The subjects were not accessible to the principal information and the study data. However, the principal treating clinician was informed of the virological test results as soon as it is available and

verified. This helped to provide useful laboratory feed-back to the clinician that would benefit the patient. The detailed information collected in the patient data collection form will be used for analysis in the future study.

2.9 Meteorological data collection and analysis

The seasonality of respiratory viruses was further analysed to determine its associations with meteorological factors such as temperature (˚C) and rainfall (mm). Meteorological data were provided by the Malaysian Meteorological Department (Sarawak branch), from three weather stations located in Kuching Airport, Bintulu Airport and Sibu Airport. The monthly data from three stations were averaged before correlation with monthly respiratory cases. The associations between meteorological conditions and respiratory viruses were evaluated using Spearman's rank correlations. A P-value < 0.05 was considered statistically significant. All analyses for meteorological data were performed using the Statistical Package for SAS for windows, version 7 (SAS, USA).

2.10 Statistical analysis

Statistical analysis was performed using SPSS version 17.0 software (IBM Corp., Armonk, NY, USA). Descriptive statistics were used to describe the demographic and clinical data. The Chi-square or Fisher exact test was employed for categorical variables. A p-value of 0.05 was considered significant.

CHAPTER 3

RESULTS

3.1 Overview of the epidemiology of severe community-acquired viral lower respiratory tract infection in children aged 5 years and below in Sarawak.

3.1.1 Epidemiological findings according to multiplex RT-PCR

A total of 1165 specimens (1135 nasal swab, 7 nasopharyngeal aspirates, and 23 ETT) were obtained from pediatric patients with ALRI between Nov 2012 and Oct 2015. Most of the patients entered the study once, but 32 patients were admitted to the study more than once. For the purposes of analysis, each hospitalization was considered to be a separate entity or episode. Table 3.1 shows the total pediatric ALRI episodes of the three hospitals recruited in this study in Sarawak with a higher recruited episodes in the first year of study compared to the second and third year. The total pediatric ALRI recruited episodes during this three year study period was as follows: 462 episodes (40%) in Bintulu hospital, 203 (17%) in SGH and 500 (43%) in Sibu Hospital. Figure 3.1 depicts the monthly pediatric ALRI recruited episodes among the three study hospitals.

Table 3.1: Total episodes recruited into study among three hospitals by year (N=1165)

	Year 1 (Nov 2012-Oct 2013)	**Year 2 (Nov 2013-Oct 2014)**	**Year 3 Nov 2014-Oct 2015)**	**Entire study (Nov 2012-Oct 2015)**
Bintulu	198 (38%)	103 (35%)	161 (46%)	462 (40%)
SGH	106 (20%)	37 (12%)	60 (17%)	203 (17%)
Sibu	216 (42%)	157 (53%)	127 (37%)	500 (43%)
Total	520 (45%)	297(26%)	348(30%)	1165 (100%)

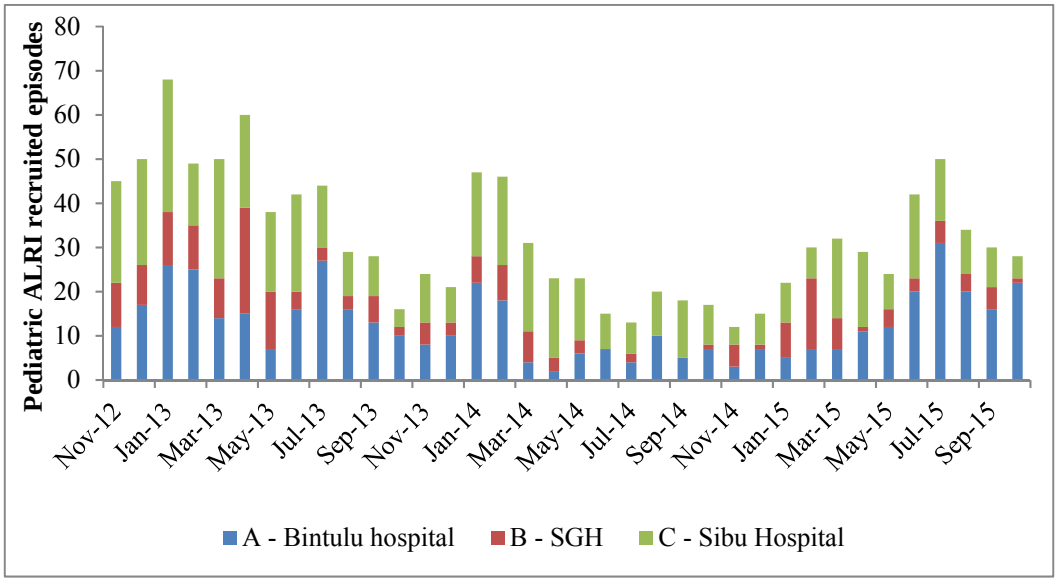


Figure 3.1: Monthly pediatric ALRI recruited episodes among three hospitals

In this study, patients were grouped by age groups: 1-12, 13-24, 25-36 months old respectively and 3-5 years old. The largest age group in our study was that of 1-12 months old with 654 episodes (56%) followed by 312 episodes (27%) who were 13-24 months old, 93 (8%) 25-36 months old and 106 (9%) 3 to 5 years old. With respect to patient gender, there were 671 (58%) males and 494 (42%) females (male: female sex ratio of 1.4). Of all pediatric ALRI recruited episodes, 989 (85%) were diagnosed with pneumonia, 79 (7%) had acute bronchiolitis, 49 (4%) had acute exacerbation of bronchial asthma, 20 (2%) were newly diagnosed asthma with concurrent respiratory tract infection, 10 (1%) had acute exacerbation of chronic lung disease, 9 (1%) had viral croup and viral wheeze respectively (Table 3.2).

Table 3.2: Characteristics and Number of Viral Detections (%) in ALRI

Characteristics	All ALRI episodes n (%)	Any Viral detection n (%)	RSV n (%)	HAdV n (%)	HRV/EV n (%)	HPIV n (%)	HMPV n (%)	HCoV n (%)	HBoV n (%)	IFV n (%)	Single virus detection n (%)	Multiple viruses detection n (%)	Negative n (%)
Total	**1165 (100)**	**902 (77.4)**	**434 (37.2)**	**340 (29.2)**	**200 (17.2)**	**104 (8.9)**	**51 (4.4)**	**27 (2.3)**	**26 (2.2)**	**22 (1.9)**	**632 (54.2)**	**270 (23.2)**	**263 (22.6)**
Gender													
Female	494 (42.4)	***366 (74.1)***[a]	174 (35.2)	133 (26.9)	76 (15.4)	44 (8.9)	21 (4.3)	13 (2.6)	11 (2.2)	12 (2.4)	256 (51.8)	110 (22.3)	128 (25.9)
Male	671 (57.6)	***536 (79.9)***	260 (38.7)	207 (30.8)	124 (18.5)	60 (8.9)	30 (4.5)	14 (2.1)	15 (2.2)	10 (1.5)	376 (56.0)	160 (23.8)	135 (20.1)
Age													
1- 12 months	654 (56.1)	***535 (81.8)***[b]	***277 (42.4)***[e]	181 (27.7)	***133 (20.3)***[e]	64 (9.8)	28 (4.3)	16 (2.4)	***9 (1.4)***[e]	12 (1.8)	370 (56.6)	165 (25.2)	119 (18.2)
13-24 months	312 (26.8)	240 (77.0)	104 (33.3)	103 (33.0)	44 (14.1)	27 (8.7)	16 (5.1)	8 (2.6)	***15 (4.8)***[e]	6 (1.9)	167 (53.5)	73 (23.4)	72 (23.1)
25-36 months	93 (8)	66 (71.0)	32 (34.4)	29 (31.2)	10 (10.8)	6 (6.5)	3 (3.2)	3 (3.2)	2 (2.1)	2 (2.1)	47 (50.5)	19 (20.4)	27 (29.0)
37-60 months	106 (9.1)	61 (57.5)	21 (19.8)	27 (25.5)	13 (12.3)	7 (6.6)	4 (3.9)	0 (0)	0 (0)	2 (1.9)	48 (45.3)	13 (12.3)	45 (42.5)
Clinical Diagnosis													
Pneumonia	989 (84.9)	***751 (75.9)***[c]	***379 (38.3)***[f]	279 (28.2)	***147 (14.9)***[f]	82 (8.3)	42 (4.2)	23 (2.3)	19 (1.9)	15 (1.5)	541 (54.7)	210 (21.2)	238 (24.1)
Acute bronchiolitis	79 (6.8)	67 (84.8)	36 (45.6)	21 (26.6)	21 (26.6)	9 (11.4)	3 (3.8)	2 (2.5)	3 (3.8)	5 (6.3)	40 (50.6)	27 (34.2)	12 (15.2)
Acute exacerbation of bronchial asthma	49 (4.2)	42 (85.7)	10 (20.4)	20 (40.8)	17 (34.7)	3 (6.1)	4 (8.2)	1 (2.0)	2 (4.1)	2 (4.1)	25 (51.0)	17 (34.7)	7 (14.3)
Newly diagnosed asthma with concurrent respiratory tract infection	20 (1.7)	19 (95)	4 (20)	11 (55)	9 (45)	2 (10)	0 (0)	0 (0)	1 (5)	0 (0)	11 (55)	8 (40)	1 (5)
Acute exacerbation of chronic lung disease	10 (0.9)	6 (60)	2 (20)	3 (30)	2 (20)	0 (0)	0 (0)	0 (0)	1 (10)	0 (0)	4 (40)	2 (20)	4 (40)
Viral wheeze	9 (0.8)	8 (88.9)	2 (22.2)	4 (44.4)	4 (44.4)	1 (11.1)	1 (11.1)	0 (0)	0 (0)	0 (0)	5 (55.5)	3 (33.3)	1 (11.1)
Viral croup	9 (0.8)	9 (100)	1 (11.1)	2 (22.2)	0 (0)	7 (77.7)	1 (11.1)	1 (11.1)	0 (0)	0 (0)	6 (66.6)	3 (33.3)	0 (0)

Data are presented as number (%).

[a] Any viral detection versus all ALRI episodes among gender: $p < 0.05$, calculated using Chi-square

[b] Any viral detection versus all ALRI episodes in age group 1-12 months: $p < 0.05$, calculated using Chi-square

[c] Any viral detection versus all ALRI episodes in clinical diagnosis of pneumonia: $p < 0.05$, calculated using Chi-square

[d] No. of virus versus all ALRI episodes among gender: $p < 0.05$, calculated using Chi-square

[e] No. of virus versus all ALRI episodes in age group 1-12 months: $p < 0.05$, calculated using Chi-square

[f] No. of virus versus all ALRI episodes in clinical diagnosis of pneumonia: $p < 0.05$, calculated using Chi-square

Abbreviations: ALRI: Acute lower respiratory infections, RSV: respiratory syncycial virus, HAdV: human adenovirus, HRV/EV: Human rhinovirus/enterovirus, HPIV: human parainfluenza viruses, HMPV: human metapneumovirus, HCoV: Human coronavairus, HBoV: Human bocavirus, IFV: Influenza virus

3.1.2 Identification of viruses associated with ALRI

A total of 1204 viruses were detected in 902 (77%) of the total 1165 hospital recruited episodes. A total of 632 (54%) episodes had a single virus detected and 270 (23%) episodes had more than one virus detected. The most frequent detected viruses were RSV (n=434, 37.2%), HAdV (n=340, 29.2%), HRV/EV (n=200, 17.2%) and HPIV (n=104, 8.9%). HMPV (n=51, 4.4%), HCoV (n=27, 2.3%), HBoV (n=26, 2.2%) and influenza viruses (n=22, 1.9%) were detected at a lower frequency. A total of 263 (23%) of the pediatric ALRI recruited episodes tested negative for respiratory viral detection in this study.

More male was detected with viral ALRI compared to female ($\lambda^2(1) = 5.46$, $p = 0.019$). Besides that, children under 1 years old were the most susceptible to respiratory viral pathogens ($\lambda^2(1) = 16.36$, $p < 0.001$). Among those age group of 1-12 months, RSV was more frequently detected (42.4%) compared to other age group. Furthermore, RSV ($P = 0.000$), HRV/EV ($p = 0.001$) and HBoV ($p = 0.025$) were significantly detected among those age group of 1-12 months. In addition, RSV and HRV were significantly detected among those with pneumonia whereas for other clinical diagnosis, HAdV and HRV were more common except for viral croup whereby 77% was detected with HPIV (Table 3.2).

The monthly positive rates varied from 40% to 93% with a mean of 74%. The virus detection rates in all tested samples were higher than 70%, except in November 2012, November 2013, July 2014, September 2014, November 2014 – March 2015 and May 2015.The positive rate was highest in the month of July and Sept 2013 and also in July 2015 when there was an increase in the detection of adenovirus episodes (Figure 3.2).

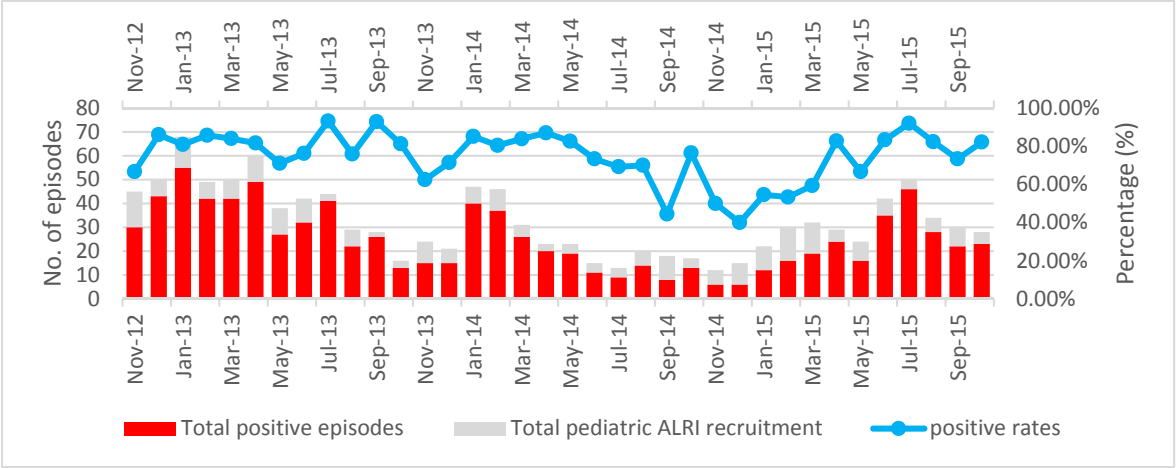


Figure 3.2: Monthly distribution and positive rates of 1165 ALRI episodes received between Nov 2012 – Oct 2015

3.1.2.1 Viral detection by hospital

As shown in Table 3.3, the total positive rate for Bintulu and SGH were 84% and 81% respectively whereas for Sibu, the positive rate was 70%. Overall, Sibu showed a lower positive rate compared to other two hospitals ($\lambda^2(2) = 30.075$, $p < 0\ .001$).

Table 3.3: Total positive and positive rates among hospitals of study by year (N=902)

	Year 1 (Nov 2012-Oct 2013)	Year 2 (Nov 2013-Oct 2014)	Year 3 Nov 2014-Oct 2015)	Entire study (Nov 2012-Oct 2015)
Bintulu	170/198 (86%)	87/103 (84%)	132/161 (82%)	389/462(84%)
SGH	90/106 (86%)	33/37 (86%)	41/60 (68%)	164/203 (81%)
Sibu	162/216 (75%)	107/157 (68%)	80/127 (63%)	349/500 (70%)
Total	422/520(81%)	227/297 (76%)	253/348(73%)	902/1165 (77%)

3.1.2.2 Viral detection by age group

All the eight group of viruses investigated in this study were detected in each age group with the exception of HCoV and HBoV which were not detected in patients 36-60 months old (Figure 3.3). RSV was significantly identified in children ≤ 1-year-old,

accounting for 51.8% (n= 277) of the positive episodes in this age group ($\lambda^2(1) = 16.601$, $p < 0.001$) . However, this declined to 43.3% (n= 104) in 13-24 months, 48.5% (n=32) in 25-36 months and 34.4% (n=21) in 37-60 months respectively. Correspondingly, the percentage of adenovirus detection increased with age according to age groups, from 33.6% (n=180) to 42.9% (n= 103) to 43.9% (n=29) and 44.3% (n= 27). However, there was no significant different of HAdV detection across age group ($\lambda^2(3) = 3.819$, $p = 0.282$) (Figure 3.3).

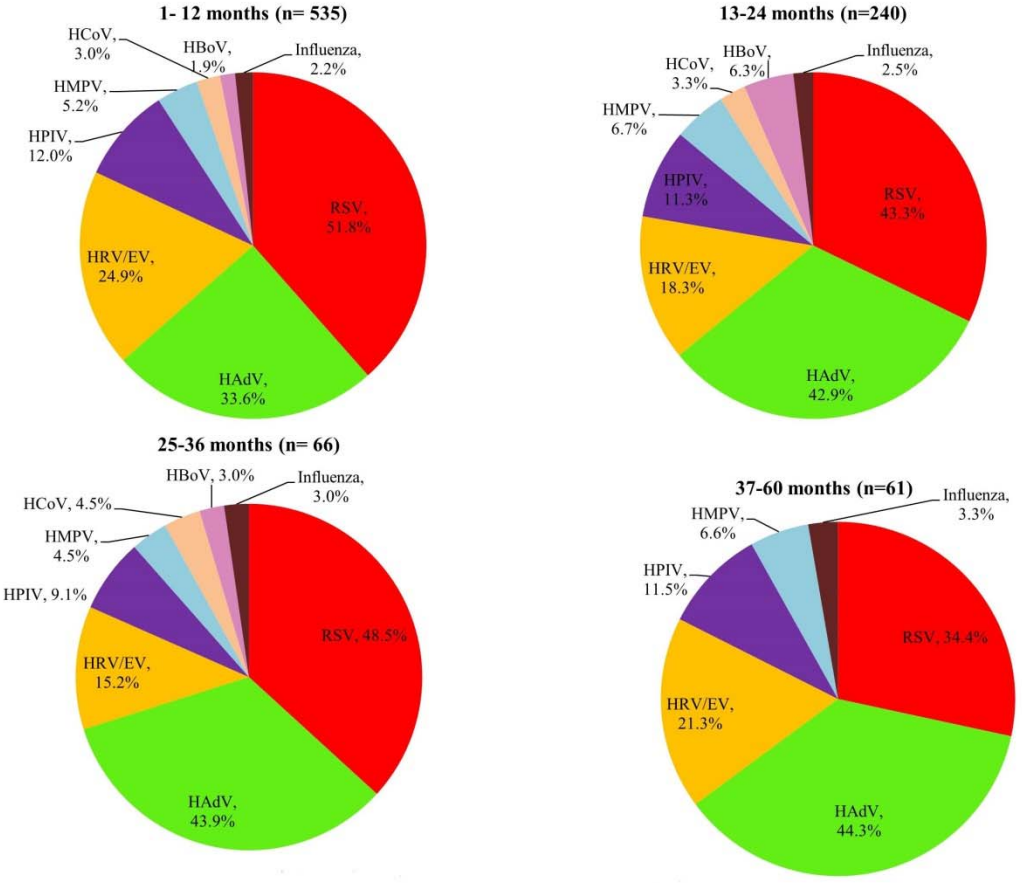


Figure 3.3: Distribution of viruses by age group

3.1.2.3 Viral distribution and clinical diagnosis of patients

As shown in Table 3.4, pneumonia is the most common clinical diagnosis made among the positive episodes, accounted for 83.3% of all positive episodes. Other clinical diagnosis of

the positive episodes included acute bronchiolitis (7.4%), acute exacerbation of bronchial asthma (4.7%) and newly diagnosed asthma with concurrent respiratory tract infection (2.1%). Less common diagnosis included acute exacerbation of chronic lung disease (0.7%), viral wheeze (0.9%) and viral croup (1%).

Table 3. 4: Incidence of positive episodes according to clinical diagnosis

Clinical Diagnosis	No. of positive episodes (n=902)	Percentage (%)
Pneumonia	751	83.3
Acute bronchiolitis	67	7.4
Acute exacerbation of bronchial asthma	42	4.7
Newly diagnosed asthma with concurrent respiratory tract infection	19	2.1
Acute exacerbation of chronic lung disease	6	0.7
Viral wheeze	8	0.9
Viral croup	9	1

As shown in Figure 3.4, pneumonia was mainly associated with RSV, HAdV and HRV representing 50.5%, 37,2% and 19.6% respectively of positive pneumonia episodes. In acute bronchiolitis positive episodes, RSV (53.7%), followed by HRV and HAdV (31.3% respectively) were the viruses frequently detected. In contrast, HAdV was the most commonly virus detected in positive episodes of acute exacerbation of bronchial asthma, acute exacerbation of lung disease, newly diagnosed bronchial asthma and viral wheeze. HRV and HPIV were the other common viruses detected in these cases. In this study, croup was caused most frequently by parainfluenza viruses, especially type 1, which accounted for 6/9 (67%) from all positive episodes with a clinical diagnosis of croup.

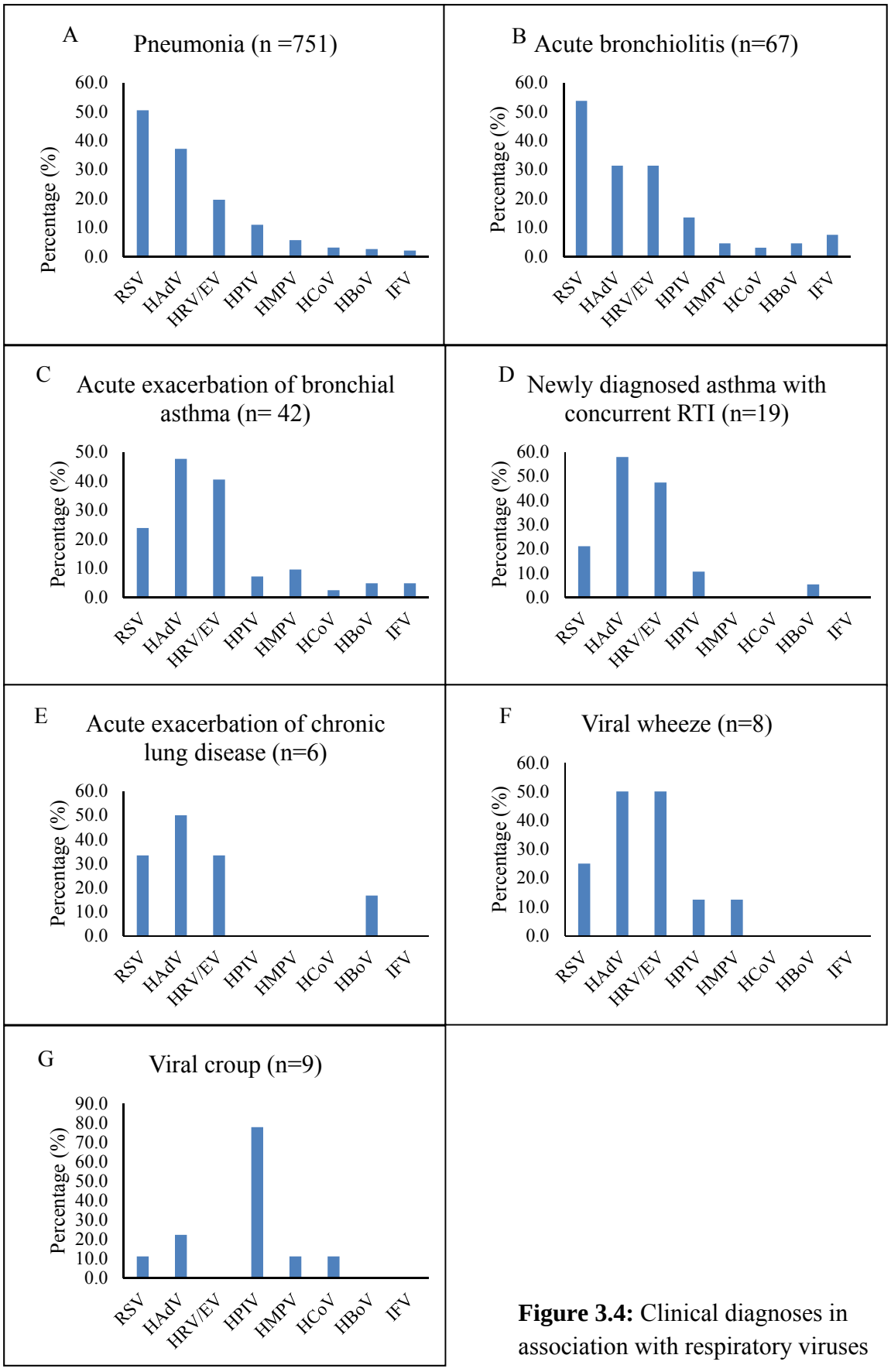


Figure 3.4: Clinical diagnoses in association with respiratory viruses

3.1.2.4 Viral distribution and typing of respiratory viruses

RSV was identified in 36.05% (434 of 1204 viruses detected) of the total viruses detected in the study followed by HAdV (28.24%, n=340), HRV/EV (16.61%, n=200), HPIV (8.64%, n=104), HPMV (4.24%, n=51), HCoV (2.24%, n=27), HBoV (2.16%, n=26) and IFV (1.83%, n=22).

Our results showed that RSV, HAdV and HRV were the most common viruses detected (approximately 80% of all viruses detected) in patients aged 5 years and below with severe ALRI admitted to PICU ward in this study. Figure 3.5 shows the overall distribution of viruses detected in this study.

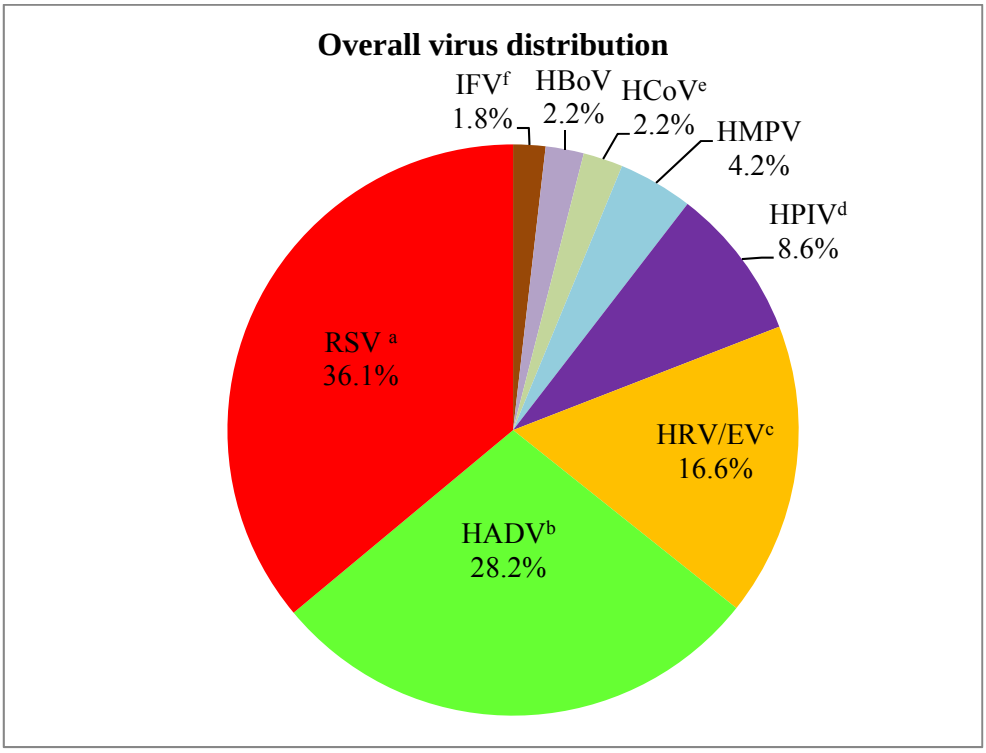


Figure 3.5: Viral etiologies of ALRI. [a] RSV A (22.6%); RSV B (13.5%), [b]HAdV B (20.1%); HAdV C (7.1%); HAdV D (0.3%), HAdV E (0.3%), HAdV F (0.5%), [C] HRV A (7.3%), HRV B (0.6%), HRV C (7.1%), EV 68 (1.2%), Coxsackievirus (0.3%); [d]HPIV-1 (3.1%), HPIV-3 (5.0%), HPIV-4(0.6%), [e] HCoV-NL63 (0.7%), HCoV-229E (0.7%), HCoV-OC43 (0.8%) [f] IAV (0.8%), IBV (0.9%), ICV (0.1%), % by 1204 viruses detected among 902 total positive episodes.

RSV was the sole viral agent detected in 32.4% (292/902) of the total positive episodes while 15.7% (142/902) were co-detected with other viruses including dual detections in 123 (13.6%) and multiple co-detections in 19 (2.1%) positive episodes. RSV positive results were further divided into antigenic subgroups A and B by sequencing. Of 434 RSV detected, 272 (63%) were identified as subgroup A and 162 (37%) as subgroup B (Table 3.5).

HAdV were detected in 37.7% (340/902) of the positive episodes in which 15.7% (142/902) episodes had a single viral detection of HAdV and 21.9% (198/902) episodes were co-detected with other viruses. All positive results were sequenced and basic local alignment search tool (BLAST) sequencing analysis showed that 71.2% (242/340) were HAdV species B (HAdV-7, n=176; HAdV-3, n=65; HAdV-11, n=1), 25.3% (86/340) were HAdV species C (HAdV-1, n= 21; HAdV-2, n= 40; HAdV-5, n=15; HAdV-6, n=10), 1% (3/340) were HAdV species D (HAdV-8, n=1; HAdV-36, n=1; HAdV-54, n-1), 1% (3/340) were HAdV species E (HAdV-4, n= 3) and 1.8% (6/340) were HAdV species F (HAdV-40, n= 1, HAdV-41, n=5). No HAdV species A and G was detected (Table 3.5).

Among the total positive episodes, HRV/EV was detected singly in 10.6% (96/902) while 11.5% (104/902) were co-detected with other viruses. 44% (88/200) of the HRVs detected were HRV-A, 3.5% (7/200) were HRV-B, 43% (86/200) were HRV-C, 7.5% (15/200) were EV-68 and 2% (4/200) were Coxsackieviruses A24, B5, B4 and B5.

HPIV was detected in 11.5% (104/902) of the positive episodes that yielded a positive viral detection. Of these, 7% (63/902) were co-detected with other viruses. HPIV-3 was the most frequently variant detected among HPIV-positive episodes, accounting for 57.7% (60/104). HPIV-1 and HPIV-4 were detected in 35.6% (37/104) and 6.7% (7/104)

respectively. Other respiratory viruses which were detected at a lower frequency are listed in Table 3.5. HPIV-2, HCoV-HKU1 and SARS-CoV were not detected in this study.

Table 3.5: Respiratory viral detections according to subtype

Viruses detected	Single virus detection (N=632), n(%)	Co-detections (N=270) n(%)	Total (N=902) n(%)
RSV-A	191(21.2)	81 (9)	272 (30.2)
RSV-B	101(11.2)	61 (6.8)	162 (18.0)
HAdV-3	22(2.4)	43 (4.8)	65 (7.2)
HAdV-7	94(10.4)	82 (9.1)	176 (19.5)
HAdV-11	1(0.1)	0.0	1 (0.1)
HAdV-1	5(0.6)	16 (1.8)	21 (2.3)
HAdV-2	14(1.6)	25 (2.8)	40 (4.4)
HAdV-5	2(0.2)	13 (1.4)	15 (1.7)
HAdV-6	1(0.1)	9 (1)	10 (1.1)
HAdV-8	0	1 (0.1)	1 (0.1)
HAdV-36	0	1 (0.1)	1 (0.1)
HAdV-54	0	1 (0.1)	1 (0.1)
HAdV-4	1(0.1)	2 (0.2)	3 (0.3)
HAdV-40	0	1 (0.1)	1 (0.1)
HAdV-41	2(0.2)	3 (0.3)	5 (0.6)
HRV-A	36(4)	52 (5.8)	88 (9.8)
HRV-B	2(0.2)	5 (0.6)	7 (0.8)
HRV-C	45(5)	41 (4.5)	86 (9.5)
EV-68	11(1.2)	4 (0.4)	15 (1.7)
Coxsackievirus	2(0.2)	2 (0.2)	4 (0.4)
HPIV-1	17(1.9)	20 (2.2)	37 (4.1)
HPIV- 2	0	0.0	0
HPIV-3	21(2.3)	39 (4.3)	60 (6.7)
HPIV-4	3 (0.3)	4 (0.4)	7 (0.8)
HMPV	28(3.1)	23 (2.5)	51 (5.7)
Influenza A	6(0.7)	4 (0.4)	10 (1.1)
Influenza B	6(0.7)	5 (0.5)	11 (1.2)
Influenza C	0	1 (0.1)	1 (0.1)
HCoV-229E	3(0.3)	6(0.7)	9 (1)
HCoV HKU1	0	0.0	0
HCoV-NL63	2(0.2)	6(0.7)	8 (0.9)
HCoV-OC43	7(0.8)	3 (0.3)	10 (1.1)
SARS-CoV	0	0.0	0
HBoV	9(1)	17 (1.9)	26 (3)

Data are presented as number (% of total positive episodes).
Abbreviations: ALRI: Acute Lower Respiratory Infections, RSV: respiratory syncycial virus, HAdV: human adenovirus, HRV/EV: Human Rhinovirus/Enterovirus, HPIV: human parainfluenza viruses, HMPV: human metapneumovirus, HCoV: Human Coronavairus, HBoV: Human Bocavirus, IFV: Influenza virus

3.1.3 Seasonal activity of respiratory viruses

Figure 3.6 depicts the seasonal distribution viruses detected throughout this study. Over the 36-month period, there were clear seasonal peaks for RSV and HAdV with some overlap. RSV activity was present throughout the 36-month period. There were 3 pronounced seasons observed: the first wave was from November 2012 to April 2013 with a peak in January to February; the second wave was from January to May 2014, also with a peak in January to February; the third peak was from March to October 2015, with a peak in June to July. HAdV showed two distinct seasons in our 36-month study. HAdV peaks after RSV in March to September 2013 followed by another peak in July-October 2015 which coincides with the RSV season. HRV and HPIV were endemic throughout the year. Although HMPV had a low detection rate in the study, a small peak was observed in April soon after the RSV peak in 2013. HCoV, HBoV and influenza viruses were sporadically detected throughout the study period (Figure 3.6).

Out of the four main viruses detected (HAdV, HRV/EV, RSV and PIV), HAdV and RSV were temporarily related. Co-detection of HAdV and RSV overlapped during the HAdV and RSV season while HRV and HPIV occurred baseline levels. It was also observed that both RSV and HAdV were detected in recruited episodes as single virus infections during non-HAdV or non-RSV seasons indicating they might act as the sole virus contributing to the disease (Figure 3.7).

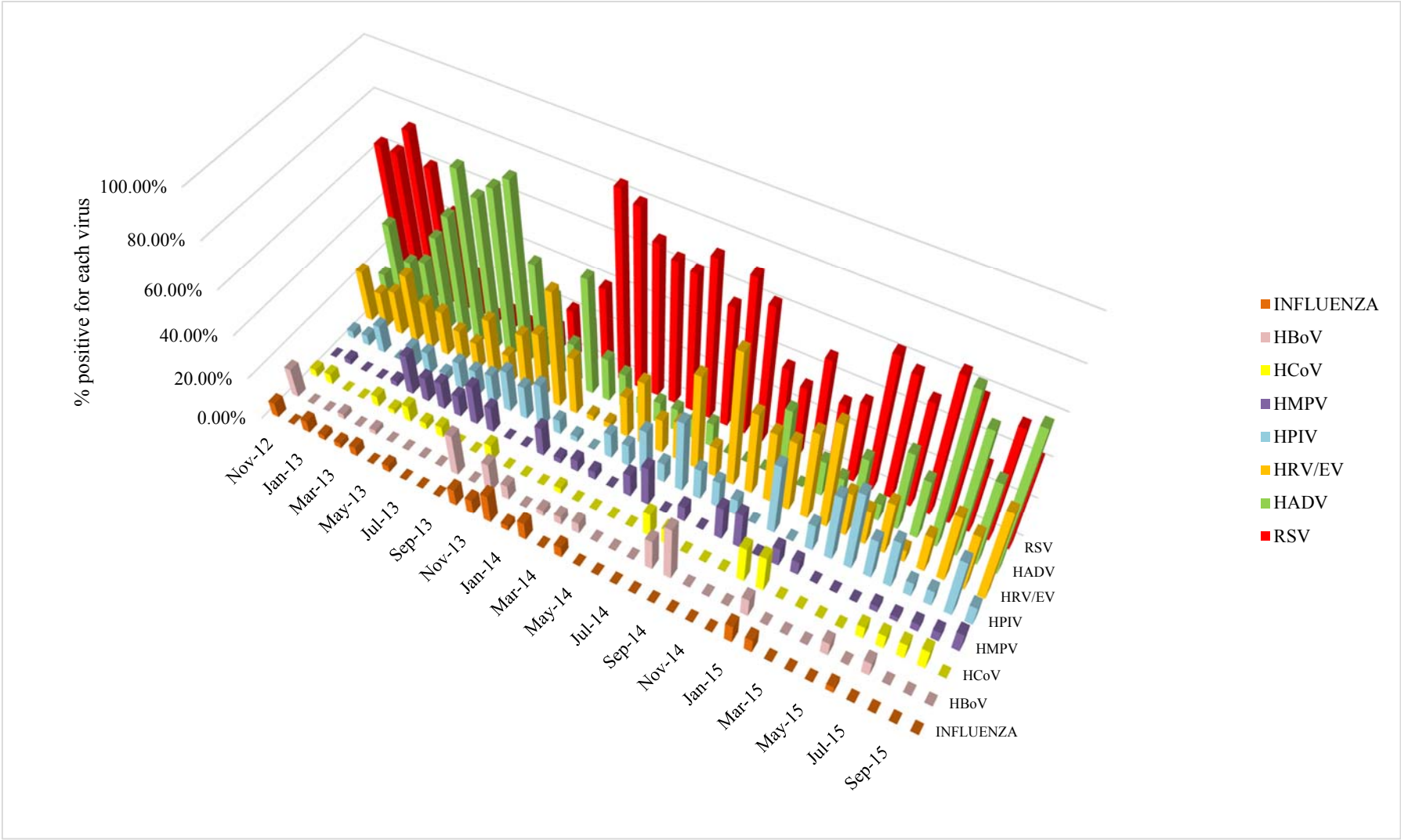


Figure 3.6: Temporal co-circulation patterns of respiratory viruses in children under 5 years old during the study period, Nov 2012- Oct 2015

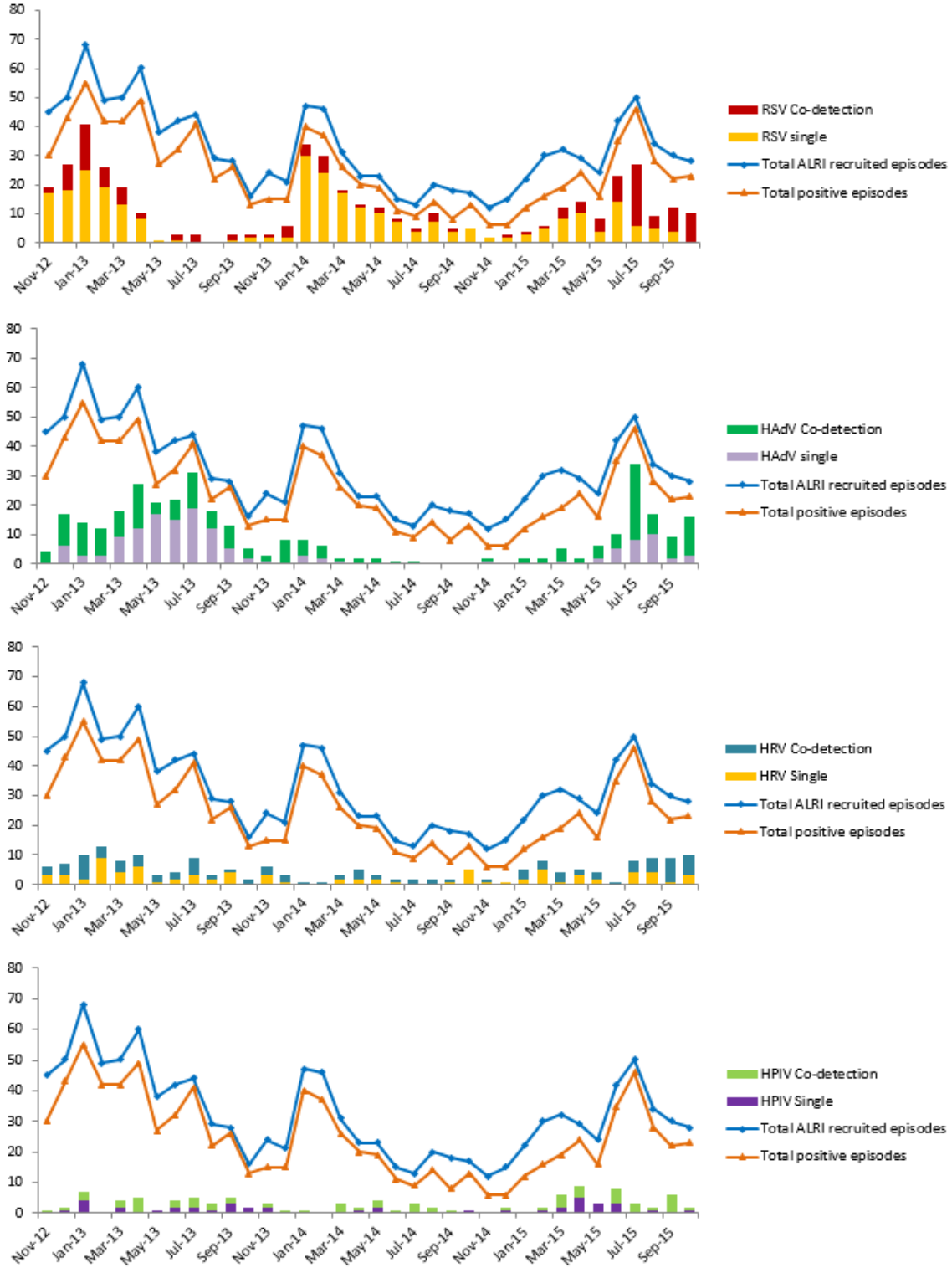


Figure 3.7: The trend of co-detection for the four main respiratory viruses across the study period.

3.1.3.1 Seasonal activity of respiratory viruses in relation to meteorological factors

The association between meteorological factors and the four main respiratory viruses detected in this study are shown in Figure 3.8. It was observed that RSV peaks after the rainy season. Pearson's correlation showed a negative correlation between RSV detections with temperature and rainfall as shown in Table 3.6. HAdV activity peaks during hot months in 2013 and 2015 and was positively associated with temperature ($r= 0.384$, $p < 0.05$). HPIV was positively associated with temperature and negatively associated with rainfall ($r= 0.406$, $p < 0.05$; $r= -0.436$, $p < 0.05$). Similar to RSV, no clear seasonal pattern in association to meteorological factors was observed for HRV.

Table 3.6: Correlation between ALRI-related hospital recruitment in children ≤ 5 years old and meteorological factors

Viruses	Monthly Mean Temperature (˚C)		Monthly Mean Rainfall (mm)	
	Correlation coefficient	P	Correlation coefficient	P
RSV	-0.135	0.433	-0.041	0.814
HAdV	0.384	***0.021**	-0.272	0.109
HRV	0.101	0.556	-0.083	0.629
HPIV	0.406	***0.014**	-0.436	***0.008**

* All variables with $p < 0.05$ are shown in bold

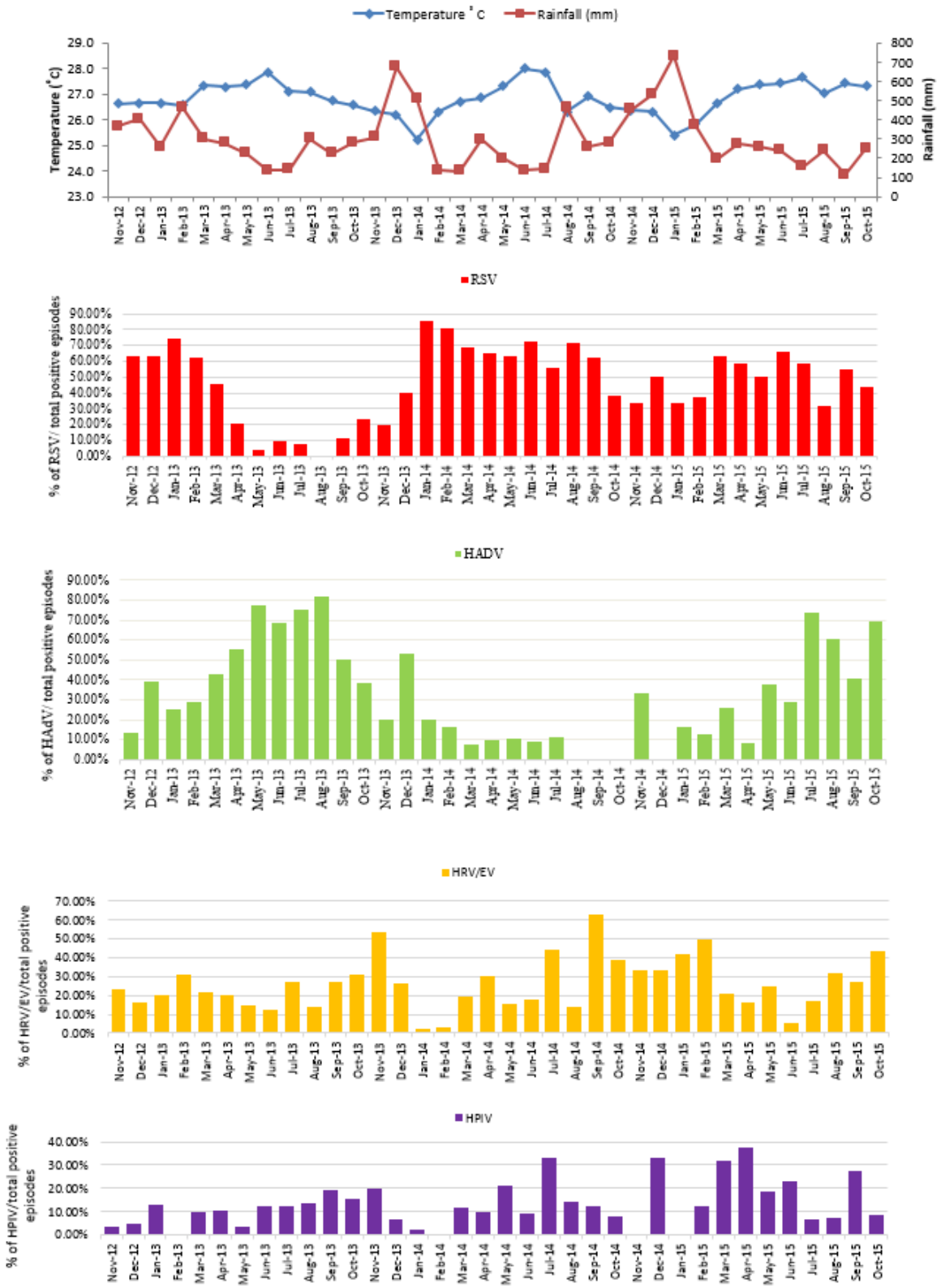


Figure 3.8: Association of meteorological factors with four main respiratory viruses

3.1.4 Multiple viral detections

Co-detection occurred in 270 episodes with the detection rates of 23% (270/1165) of all pediatric recruited episodes and 30% (270/902) of positive episodes. Among the 270 episodes, 240 had dual detections, 28 had three viruses detected and two had four viruses detected. Co-detection was observed with one or more of these four viruses (HAdV, RSV, HRV and HPIV) with the exception of one episode where HBoV is co-detected with IFV without involving the four viruses mentioned. These four viruses accounted for 91.5% of the total 902 positive episodes (Figure 3.9). The most prevalent co-detections were RSV with HAdV (n=90) followed by HAdV with HRV (n=60) and RSV with HRV (n=33) (Figure 3.8). Table 3.7 shows a detailed summary of the interaction of viruses in positive episodes in which co-detections were observed.

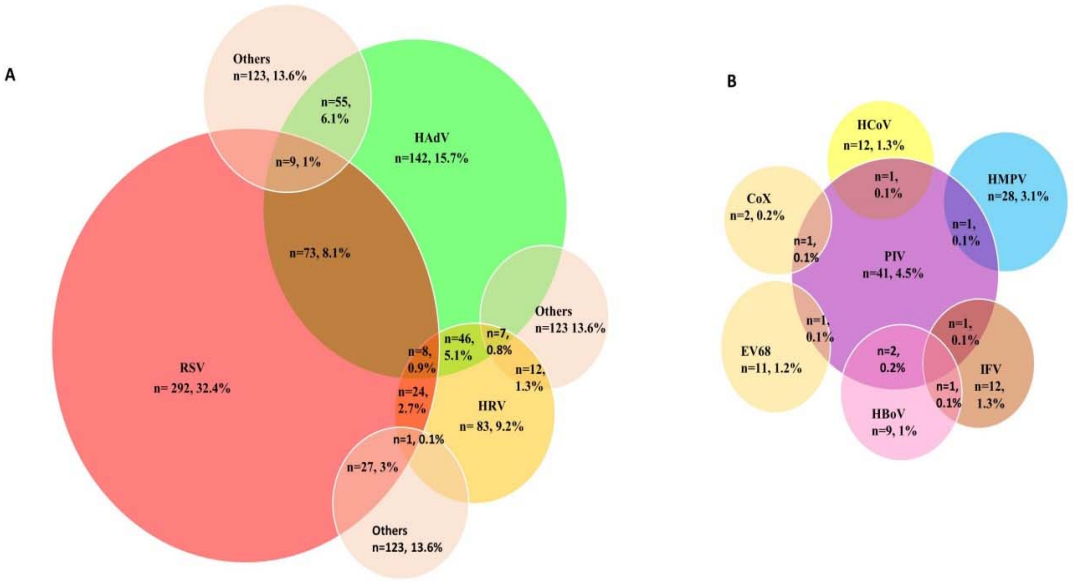


Figure 3.9: Association of co-detected viruses in ALRI episodes.
% shown by total positive ALRI episodes (n=902). Panel A describes the association between three main viruses involved in this study (HAdV, RSV, HRV); Panel B describes the association of co-detection between HPIV among other viruses (n=124)

Table 3.7: Number of Specific Viruses Co-detection combinations among ALRI episodes in children under 5 years old

Virus type	Co-detected virus type																				
	HAdV					RSV (n= 90)		HRV (n=61)			EV (n=4)	HPIV (n= 31)			HMPV (n= 21)	HBoV (n=8)	HCoV (n=6)			IFV (n=6)		
Total HAdV co-detection episodes (n=198)	HAdV-B	HAdV-C	HAdV-D	HAdV-E	HAdV-F	RSV-A	RSV-B	HRV-A	HRV-B	HRV-C		HPIV-1	HPIV-3	HPIV-4			HCoV-NL63	HCoV-OC43	HCoV-229E	IAV	IBV	ICV
HAdV B (n=125)	ND	ND	ND	ND	ND	28	27	16	1	14	2[a]	4	20		16	5	2	1	2	3	1	1
HAdV C (n=64)	ND	ND	ND	ND	ND	16	13	13	2	13	2[b]	ND	6	1	4	3	ND	ND	ND	ND	ND	ND
HAdV D (n=3)	ND	ND	ND	ND	ND	ND	1	1	ND	ND	ND	ND	ND	ND	ND	ND	1	ND	ND	ND	1	ND
HAdV E (n=2)	ND	ND	ND	ND	ND	1	1	ND	ND	ND	ND	ND	ND	ND	1	ND	ND	ND	ND	ND	ND	ND
HAdV F (n=4)	ND	ND	ND	ND	ND	ND	3	1	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	HAdV (n= 90)					RSV		HRV (n= 33)			EV (n=0)	HPIV (n= 23)			HMPV (n= 1)	HBoV (n=5)	HCoV (n=10)			IFV (n=1)		
RSV co-detection episodes (n=142)	HAdV-B	HAdV-C	HAdV-D	HAdV-E	HAdV-F	RSV-A	RSV-B	HRV-A	HRV-B	HRV-C		HPIV-1	HPIV-3	HPIV-4			HCoV-NL63	HCoV-OC43	HCoV-229E	IAV	IBV	ICV
RSV A (n=81)	28	16	ND	1	ND	ND	ND	9	ND	10	ND	7	9	1	1	3	3	1	2	ND	ND	ND
RSV B (n= 61)	27	13	1	1	3	ND	ND	9	1	4	ND	3	2	1	ND	2	1	1	2	ND	ND	1
	HAdV (n= 64)					RSV (n= 33)		HRV			EV (n=0)	HPIV (n= 13)			HMPV (n= 1)	HBoV (n=4)	HCoV (n=5)			IFV (n=2)		
HRV/EV Total (n=104)	HAdV-B	HAdV-C	HAdV-D	HAdV-E	HAdV-F	RSV-A	RSV-B	HRV-A	HRV-B	HRV-C		HPIV-1	HPIV-3	HPIV-4			HCoV-NL63	HCoV-OC43	HCoV-229E	IAV	IBV	ICV
HRV- A (n=52)	16	13	1	ND	1	9	9	ND	ND	ND	ND	2	5	1	ND	2	ND	ND	ND	ND	1	ND
HRV- B (n=5)	1	2	ND	ND	ND	ND	1	ND	ND	ND	ND	ND	3		1	1	ND	1	ND	ND	ND	ND
HRV- C (n=41)	14	13	ND	ND	ND	10	4	ND	ND	ND	ND	ND	ND	ND	ND	ND	3	ND	ND	1	ND	ND
EV- 68 (n=4)	1	2	ND	ND	ND	ND	ND	ND	ND	ND	ND	1	ND	ND	ND	1	ND	ND	1	ND	ND	ND
Cox (n=2)	1	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	1[c]	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	HAdV (n= 31)					RSV (n= 23)		HRV (n= 11)			EV (n=2)	HPIV			HMPV (n= 2)	HBoV (n=2)	HCoV (n=3)			IFV (n=2)		
HPIV Total (63)	HAdV-B	HAdV-C	HAdV-D	HAdV-E	HAdV-F	RSV-A	RSV-B	HRV-A	HRV-B	HRV-C		HPIV-1	HPIV-3	HPIV-4			HCoV-NL63	HCoV-OC43	HCoV-229E	IAV	IBV	ICV
HPIV- 1 (n=20)	4	ND	ND	ND	ND	7	3	2	ND	ND	2[d]	ND	ND	ND	1	1	ND	ND	1	ND	1	ND
HPIV- 3 (n= 39)	20	6	ND	ND	ND	9	2	5	3	ND	ND	ND	ND	ND	1	1	2	ND	ND	1	ND	ND
HPIV- 4 (n=4)	ND	1	ND	ND	ND	1	1	1	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

Table 3.7 continued

Virus type	Co-detected virus type																					
	HAdV (n= 21)					RSV (n= 1)		HRV (n= 1)			EV (n=0)	HPIV (n=2)			HMPV	HBoV (n=0)	HCoV (n=0)			IFV (n=0)		
Total HMPV co-detection episodes (n=23)	HAdV-B	HAdV-C	HAdV-D	HAdV-E	HAdV-F	RSV-A	RSV-B	HRV-A	HRV-B	HRV-C		HPIV-1	HPIV-3	HPIV-4			HCoV-NL63	HCoV-OC43	HCoV-229E	IAV	IBV	ICV
	16	4	ND	1	ND	1	ND	ND	1	ND	ND	1	1	ND	ND	ND	ND	ND	ND	ND	ND	ND
	HAdV (n= 7)					RSV (n= 5)		hRV (n= 3)			EV (n=1)	HPIV (n=2)			HMPV (n=0)	HBoV	HCoV (n=1)			IFV (n=1)		
Total HBoV co-detection episodes (n=17)	HAdV-B	HAdV-C	HAdV-D	HAdV-E	HAdV-F	RSV-A	RSV-B	HRV-A	HRV-B	HRV-C		HPIV-1	HPIV-3	HPIV-4			HCoV-NL63	HCoV-OC43	HCoV-229E	IAV	IBV	ICV
	5	3	ND	ND	ND	3	2	2	1	ND	1[b]	1	1	ND	ND	ND	ND	ND	1	ND	1	ND
	HAdV (n= 6)					RSV (n= 10)		HRV (n= 4)			EV (n=1)	HPIV (n=3)			HMPV (n=0)	HBoV (n=1)	HCoV			IFV (n=0)		
Total HCoV Co-detection episodes (n=15)	HAdV-B	HAdV-C	HAdV-D	HAdV-E	HAdV-F	RSV-A	RSV-B	HRV-A	HRV-B	HRV-C		HPIV-1	HPIV-3	HPIV-4			HCoV-NL63	HCoV-OC43	HCoV-229E	IAV	IBV	ICV
HCoV-NL63 (n=6)	2	ND	1	ND	ND	3	1	ND	ND	3	ND	ND	2	ND	ND	ND	ND	ND	ND	ND	ND	ND
HCoV-OC43(n=3)	1	ND	ND	ND	ND	1	1	ND	1	ND	ND	ND	ND	ND	ND	1	ND	ND	ND	ND	ND	ND
HCoV-229E (n=6)	2	ND	ND	ND	ND	2	2	ND	ND	ND	1	1	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	HAdV (n= 6)					RSV (n= 1)		HRV (n= 2)			EV (n=0)	HPIV (n=2)			HMPV (n=0)	HBoV (n=1)	HCoV (n=0)			IFV		
Total IFV Co-detection episodes (n=10)	HAdV-B	HAdV-C	HAdV-D	HAdV-E	HAdV-F	RSV-A	RSV-B	HRV-A	HRV-B	HRV-C		HPIV-1	HPIV-3	HPIV-4			HCoV-NL63	HCoV-OC43	HCoV-229E	IAV	IBV	ICV
IAV (n=4)	3	ND	ND	ND	ND	ND	ND	ND	ND	1	ND	ND	1	ND	ND	ND	ND	ND	ND	ND	ND	ND
IBV (n=5)	1	ND	1	ND	ND	ND	ND	1	ND	ND	ND	1	ND	ND	ND	1	ND	ND	ND	ND	ND	ND
ICV (n=1)	1	ND	ND	ND	ND	ND	1	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

Data are presented as number of detections.
Co-detection indicates virus was detected in combination with at least one other viruses.
Abbreviations: ALRI: Acute lower respiratory infections, RSV: respiratory syncycial virus, HAdV: human adenovirus, HRV/EV: Human rhinovirus/enterovirus, HPIV: human parainfluenza viruses, HMPV: human metapneumovirus, HCoV: Human coronavairus, HBoV: Human bocavirus, IFV: Influenza virus* 1 EV68 & 1 Coxsackievirus B5; b* EV68; c* Coxsackievirus A24; d* 1 EV 68 & 1 coxsackievirus A24
ND: Not detectable

3.2 Respiratory Syncytial Virus (RSV)

3.2.1 RSV distribution by age and gender

RSV was detected by multiplex RT-PCR in 434 (48.1%) positive episodes, making it the most prevalent virus detected among 902 positive episodes detected in this study. RSV was detected mainly in younger infants. As shown in Figure 3.10, RSV was by far the most commonly identified virus in children ≤ 1 year old, accounting for 51.8% (n= 277) of the total positive episodes in this age group. However, this declined to 43.3% (n= 104) in 13-24 months, 48.5% (n=32) in 25-36 months and 34.4% (n=21) in 37-60 months. Figure 3.10 shows a summary of these results by subgroup distribution. Among the 434 episodes, the majority are male patients as shown in Table 3.8. The male to female ratio was 1.5:1 (χ^2= 1.51, p= 0.219).

Table 3.8: Demographic characteristics of patient recruitment

Characteristics	Positive Episodes	RSV Positive	RSV Positivity by hospital[a]		
			SGH	Sibu	Bintulu
Total	n=902 (%)	n=434 (%)	n= 63(%)	n=182(%)	n=189(%)
Gender					
Female	366 (40.6)	174 (47.5)	32 (18.4)	78 (44.8)	64 (36.8)
Male	536 (59.4)	260 (48.5)	31 (11.9)	104 (40.0)	125 (48.1)
Age groups (Month)					
1- 12 months	535 (59.3)	***277 (51.8)[b]***	46 (16.6)	103 (37.2)	128 (46.2)
13-24 months	240 (26.6)	104 (43.3)	10 (9.6)	48 (46.2)	46 (44.2)
25-36 months	66 (7.3)	32 (48.5)	3 (9.4)	19 (59.4)	10 (31.3)
37-60 months	61 (6.8)	21 (34.4)	4 (19.0)	12 (57.1)	5 (23.8)

[a] Number (%) of RSV-positive episodes in each characteristics/ total positive episodes of each characteristics
[b] RSV positive episodes by age groups in 1-12 months: $p < 0.05$, calculated using Chi-square test

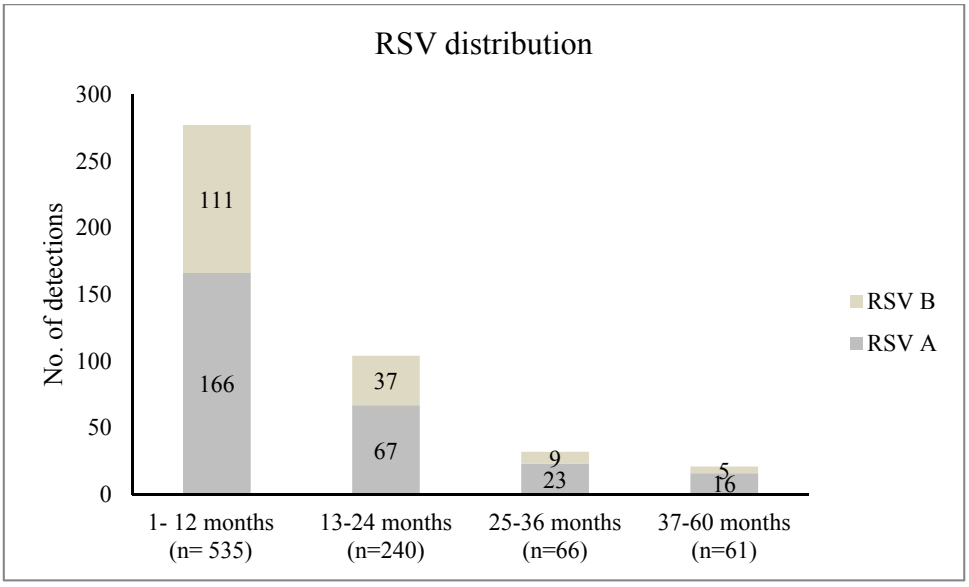


Figure 3.10: Distribution of RSV subgroup by age group

3.2.2 RSV Distribution by hospital

During the three-year study period (November 2012 – October 2015), children under 5 years of age with acute viral lower respiratory tract infections were admitted to PICU ward in either one of the three study hospitals in Sarawak. There were 1165 episodes of ALRI documented. The pediatric ALRI recruited episodes in SGH was 17.4% (203/1165) while Sibu and Bintulu showed a higher recruitment rate of 42.9% (500/1165) and 39.7% (462/1165) respectively. 18.2% (164 /902) positive episodes were detected in SGH, while 38.7% (349/902) positive episodes were detected in Sibu Hospital. 43.1% (389/902) of positive episodes were detected in Bintulu Hospital. Of these, 434 were detected with RSV by using multiplex RT-PCR. The distribution of RSV among the three study hospitals is shown in Table 3.9. The RSV positivity detection ranged from 38.4% to 52.1% among the three hospitals.

Table 3.9: Respiratory syncytial virus positivity by hospital

Hospital	Total positive episodes		RSV positivity	
	n= 902	%	n=434	%
SGH	164	18.2	63	38.4
Sibu Hospital	349	38.7	182	52.1
Bintulu Hospital	389	43.1	189	48.6

3.2.3 RSV seasonality

Looking into the total distribution of RSV based on month, RSV was more frequently detected in the period between January and March with the highest percentage of 73.83% in the month of January followed by 65.26% in February. This result is shown in Table 3.10. This study revealed some patterns in RSV detection over the 3-year study period. The distribution of RSV-positive episodes changed over time. Analysis of monthly distribution of each year of the study showed a trend for an earlier appearance and an overall greater prevalence of the epidemics during the first and second season. However, there was a later onset and lower prevalence during the third season. As mentioned earlier in the previous section, there were 3 pronounced waves of RSV observed: the first wave was from November 2012 to April 2013 with peak in January to February; the second wave was from January to May 2014, also with a peak in January to February; the third wave showed a later onset from March to October 2015, with a peak in June to July (Figure 3.11). Interestingly, a similar epidemiological pattern of RSV was observed among the three study hospitals across the 36-months study period. The monthly prevalence of RSV showed clear inter-epidemic periods in the three study hospitals (Figure 3.12).

Table 3.10: Total RSV distribution by month/year

	1st season (2012-2013)	2nd season (2013-2014)	3rd season (2014-2015)	Whole study period	Total positive episodes	Percentage
Nov	19	3	2	24	51	47.06%
Dec	27	6	3	36	64	56.25%
Jan	41	34	4	79	107	73.83%
Feb	26	30	6	62	95	65.26%
Mar	19	18	12	49	87	56.32%
Apr	10	13	14	37	93	39.78%
May	1	12	8	21	62	33.87%
Jun	3	8	23	34	78	43.59%
Jul	3	5	27	35	96	36.46%
Aug	0	10	9	19	64	29.69%
Sep	3	5	12	20	56	35.71%
Oct	3	5	10	18	49	36.73%
Grand Total	155	149	130	434	902	

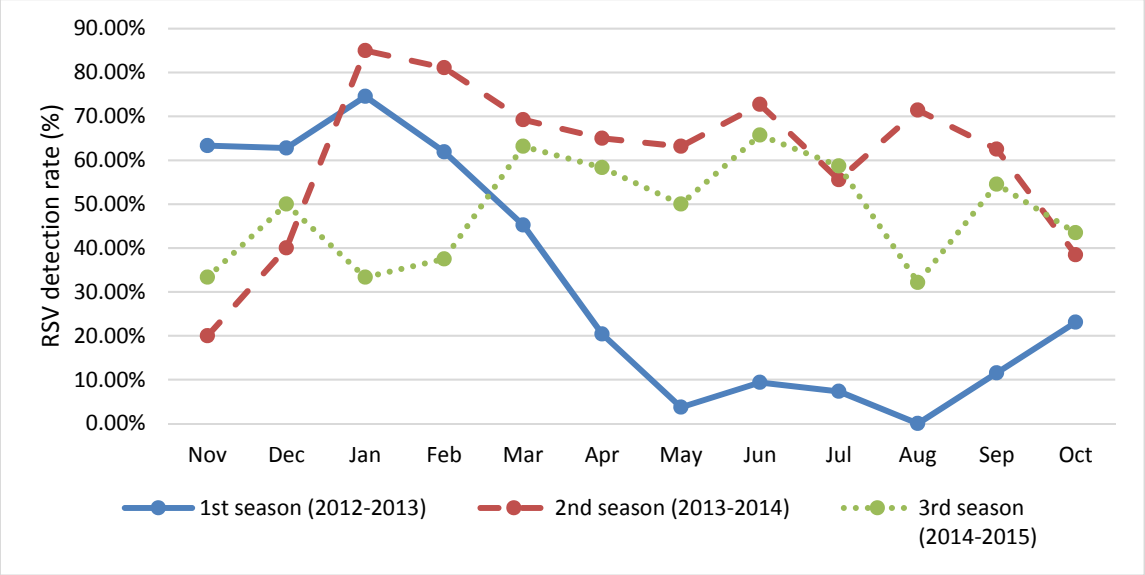


Figure 3.11: Monthly trends of RSV infection in Sarawak during each of the three consecutive epidemic seasons

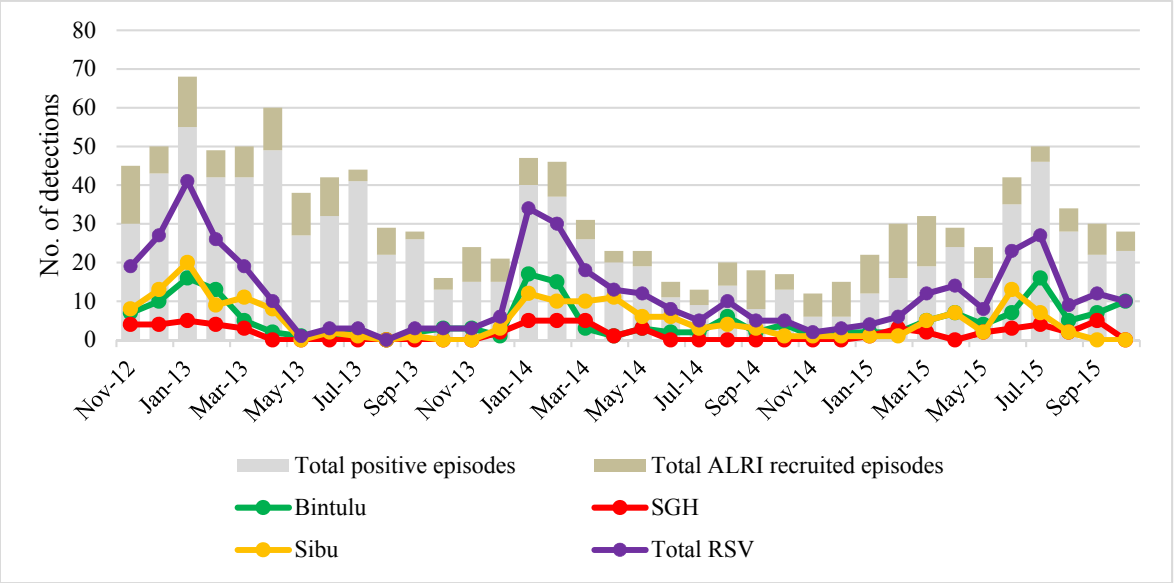


Figure 3.12: Monthly trends of RSV detection in the three study hospitals

3.2.3.1 RSV seasonality and meteorological factors

There was a clear seasonal trend for RSV observed with annual peaks occurring in the months of January and February of the 2012-2013 and 2013-2014 collection seasons which coincided with the rainy season or just after the onset of the rains. However, there was a delay in the RSV peak in the 2014-2015 season which peaked in June – July (Figure 3.13). The association of RSV with meteorological factors is less clear in our study due to the year to year variation in RSV peak activity. No association with rainfall and temperature was found.

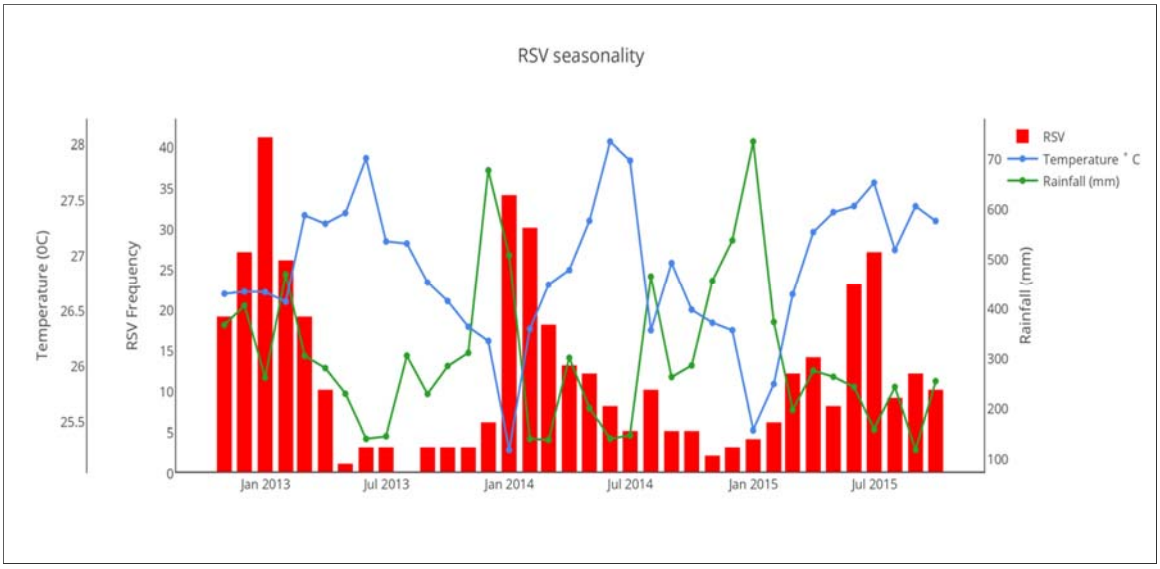


Figure 3.13: RSV seasonality in association with meteorological factors

3.2.4 RSV antigenic group

RSV is classified into antigenic subgroups A and B based on antigenic and genetic variability (Peret *et al.*, 2000). All positive PCR results were sequenced and basic local alignment search tool (BLAST) sequencing analysis showed that 63% (272/434) were RSV-A, while RSV-B was found in 37% (162/434) of the total RSV positive episodes. Although RSV-A prevailed over RSV-B, the analysis showed that there was certain variability in certain seasons. Both strains co-circulate during the 36-months study period but their numbers fluctuated from epidemic to epidemic; RSV-B was dominant in the first epidemic season (2012-2013) however, there was a shift into RSV-A predominance in the next two epidemic seasons (2013-2014 and 2014-2015). Overall, RSV-A was more prevalent (Table 3.11).

Table 3.11: RSV subgroup predominance (Nov 2012 – Oct 2015)

Season/Year	RSV-A	RSV-B	Total	Predominance
1st season (2012-2013)	39	116	155	B (75%)
2nd season (2013-2014)	139	10	149	A (93%)
3rd season (2014-2015)	94	36	130	A (72%)
Total	272	162	434	A (63%)

As mentioned previously, the samples were collected from three study hospitals in Kuching, Sibu and Bintulu. The distribution of RSV subgroup among hospitals was depicted in Figure 3.14. It was observed that in the three study hospitals, the distribution of RSV subgroup was similar throughout with RSV-B predominantly detected in the first season from November 2012 until April 2013 with a peak in January. The predominant RSV-B subgroup shifted to RSV-A in the second (2013-2014) and third season (2014-2015). However, during the 2013-2014 season, only 10 RSV-B was detected among 149 RSV positive episodes which accounted 7% of the total RSV detection. RSV-B was found to co-circulate to a lesser extend with RSV-A in the 2014-2015 season among the three hospitals. The distribution of RSV group in three hospitals are listed in Table 3.12.

Table 3.12: RSV group predominance by hospital

Hospitals	RSV –A	RSV-B	Total
Bintulu Hospital	123 (65%)	66 (35%)	189
SGH	36 (57%)	27 (43%)	63
Sibu Hospital	113 (62%)	69 (38%)	182

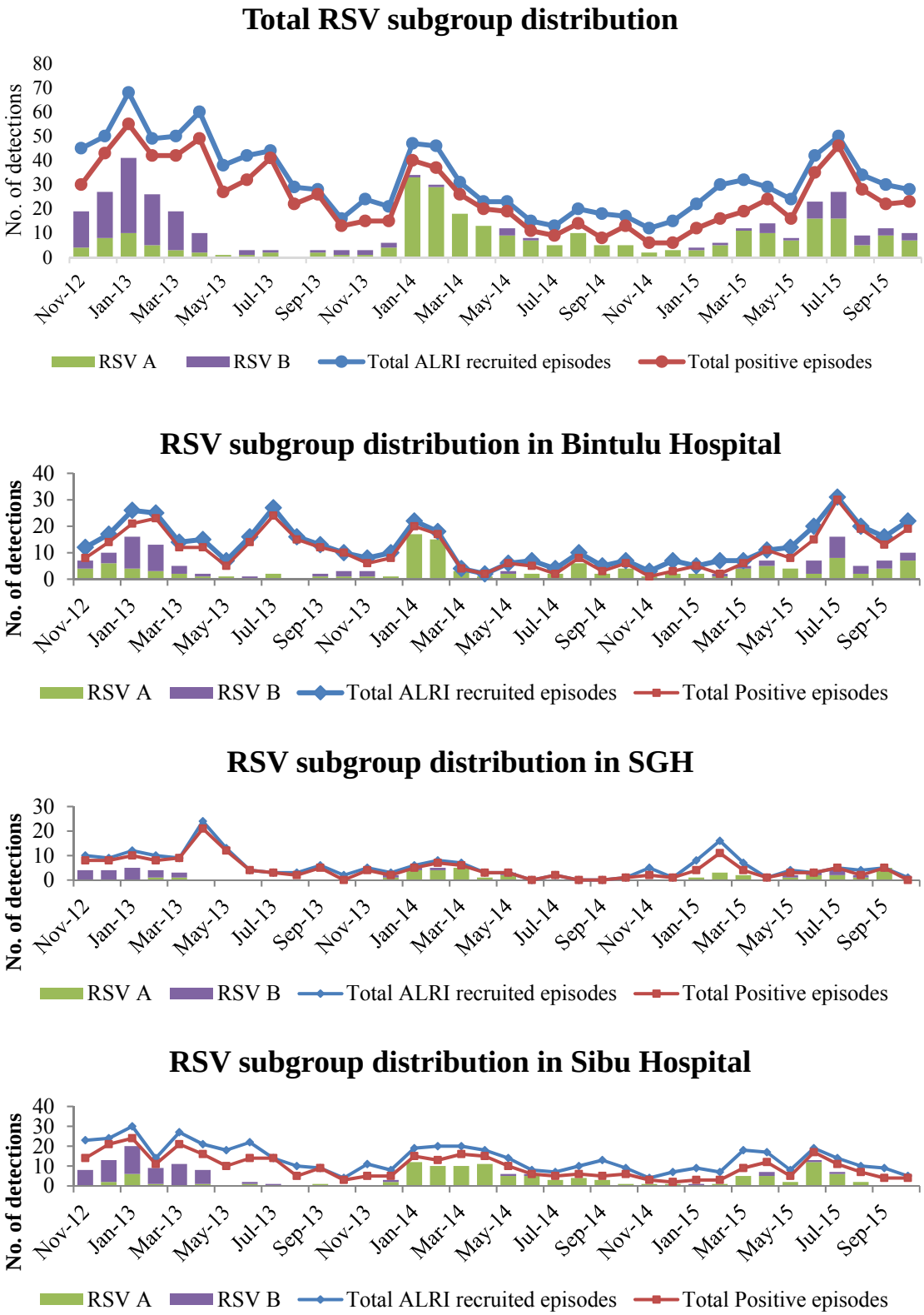


Figure 3.14: Monthly distribution of subgroup RSV-A/RSV-B over the study period by hospital. In all three study hospitals, RSV seasonality appears to be similar.

3.2.5 RSV and co-detection

RSV was the most common virus detected in this study which accounted for 48.1% (434/902) of the total positive episodes. A total of 32.7% (142/434) of all RSV positive episodes were co-detected with at least one other respiratory virus while RSV alone was detected in 67% (292/434) episodes. Co-detection between RSV and HAdV was the most frequent (73 episodes) observation followed by RSV and HRV (24 episodes). Other details of RSV co-detection are listed in Table 3.13 below.

Table 3.13: Distribution of RSV and co-detection with other respiratory viruses

Respiratory Viruses	Number of Episodes	Percentage (%)
RSV Single	292	67.3
RSV + HAdV	73	16.8
RSV + HRV	24	5.5
RSV + HPIV	16	3.7
RSV + HCoV	5	1.2
RSV + HBoV	4	1
RSV + HMPV	1	0.2
RSV + HAdV + HRV	8	1.8
RSV + HAdV + HPIV	5	1.2
RSV + HAdV + HCoV	2	0.5
RSV + HAdV + IFV	1	0.2
RSV + HPIV + HCoV	1	0.2
RSV + HAdV + HBoV + HCoV	1	0.2
RSV + HRV + HPIV + HCoV	1	0.2
Total	**434**	**100**

The monthly distributions of single and multiple viruses detection of RSV is shown in Figure 3.15. Since HAdV was the most frequent viruses co-detected with RSV, we investigated if there were any significant patterns observed for the RSV/HAdV co-detection. It was observed that RSV mostly appeared to be singly detected across the timeline which might indicates that RSV itself as the potential causative agent of ALRI during the epidemic

season. In addition, most of the RSV + HAdV co-detection occured during overlaps of the RSV and HAdV seasons as shown in Figure 3.7.

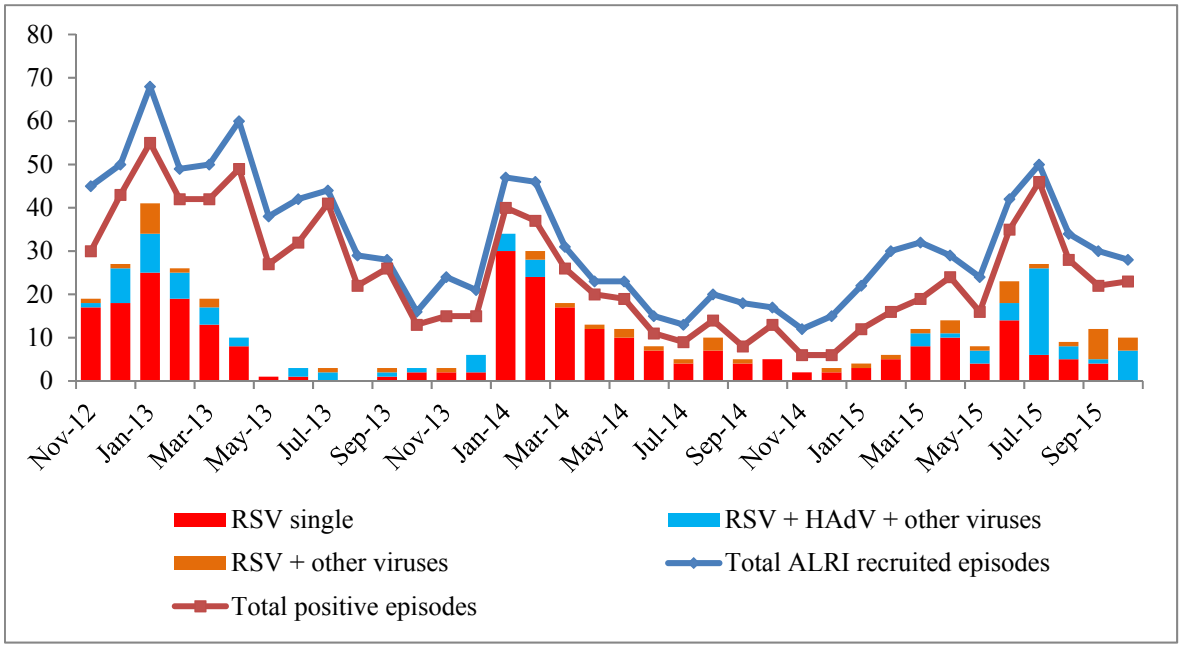


Figure 3.15: Monthly distributions of RSV and RSV co-detection

3.3 Human adenovirus (HAdV)

3.3.1 Optimization of multiplex PCR assay for the detection of Human adenovirus and Human bocavirus

We selected four samples as shown in Table 3.14 which were previously detected either with HBoV positive or/and HAdV positive for the optimization protocol. It was observed that there was no primer cross-reactivity with both HAdV and HBoV correctly detected in the multiplex PCR assay (Figure 3.16). Panel A in Figure 3.17 showed the first round PCR amplification results of HAdV and HBoV. Sample 1 was previously detected with both HAdV and HBoV and was seen with the appearance of two bands with HAdV of 562bp and HBoV of 354bp in size. HBoV was also previously detected in Sample 2 and was found being positive in this gel image with the size of 354bp. Sample 3 was weakly positive as shown in this gel image while Sample 4 was not detected in the first round of PCR. Panel B showed the nested PCR result for HAdV. All samples 1 to 4 were found HAdV positive in this gel image with the size of 349bp.

A total of 163 samples which were previously negative for HAdV using primers described by Buecher and colleagues were repeated using previously published HAdV nested PCR primers (Cardosa et al., 1999) in the modified multiplex assay. From this total 34 (20.86%) were found to be HAdV positive using the modified multiplex protocol.

Table 3.14: Viruses identified in selected samples for Multiplex 5 optimization

Sample	Patient's ID	Sequencing Results (BLAST)
1	R2/1	HMPV & HBoV, HAdV B (type 3)
2	R19/1	HBoV, EV 68, HAdV C (type 2)
3	R1/1	HAdV B (type 3)
4	13/4/1 *	HAdV C (type 2)

* Denotes non-respiratory samples

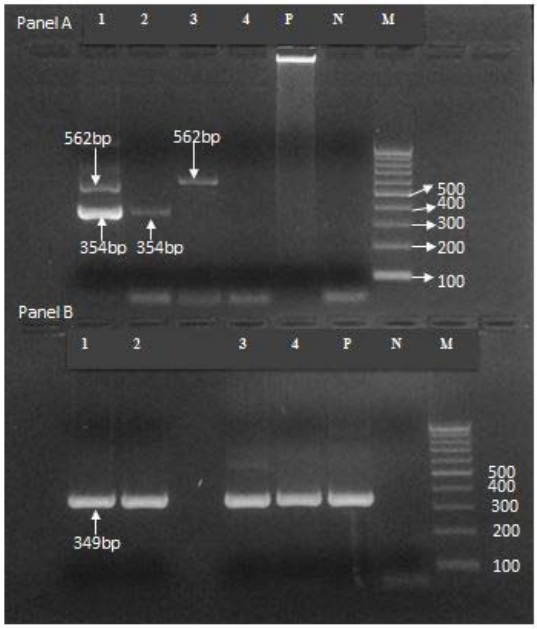


Figure 3.16: Agarose Gel Electrophoresis Analysis of Nested PCR

Panel A: Multiplex 5 first round amplification results of HAdV and HBoV
Panel B: Nested PCR for HAdV
M: DNA 100 bp marker, N: water control, P: positive control, 1-2: HAdV and HBoV positive samples, 3-4: HAdV positive samples

3.3.2 HAdV distribution by age and gender

From November 2012 through October 2015, a total of 1165 pediatric ALRI episodes were recruited into our study from three study hospitals. One or more respiratory viruses were found in 902 (77.4%) of the total hospital recruited episodes. Human adenoviruses (HAdV) were detected in 37.7% (340/902) of total positive episodes. It appears to be the second leading cause of ALRI among children less than five years old in our study.

Table 3.15 shows demographic features of the study population. Overall, HAdV positive episodes accounted for 33.8%, 42.9%, 43.9% and 44.3%, for age groups 1-12 months, 13-24 months, 25-36 months and 37-60 months respectively (Table 3.16). It was observed that HAdV detection rate increased across age group. However, they are not

statistically significant difference among age groups (χ^2= 3.819, p= 0.282). More male than female was detected with HAdV. The male to female ratio for HAdV was 1.6 (χ^2= 2.12, p= 0.145).

Table 3.15: Demographic characteristics of patient recruitment

Characteristics	All	HAdV Positive	HAdV Positivity by hospital		
			SGH	Sibu	Bintulu
Total	n=902 (%)	n=340 (%)	67 (%)	108 (%)	165 (%)
Gender					
Female	366 (40.6)	133 (36.3)	25 (18.8)	56 (42.1)	52 (39.1)
Male	536 (59.4)	207 (38.6)	42 (20.3)	52 (25.1)	113 (54.6)
Age groups (Month)					
1- 12 months	535 (59.3)	181 (33.8)	39 (21.5)	45 (24.9)	97 (53.6)
13-24 months	240 (26.6)	103 (42.9)	15 (14.6)	38 (36.9)	50 (48.5)
25-36 months	66 (7.3)	29 (43.9)	6 (20.7)	12 (41.4)	11(37.9)
37-60 months	61 (6.8)	27 (44.3)	7 (25.9)	13 (48.1)	7 (25.9)

Table 3.16: Distribution of HAdV in different age groups

Age groups (Months)	Total episodes	HAdV-positive	% of total (by age group)	P-value*
1- 12 months	535	181	33.8	0.200
13-24 months	240	103	42.9	0.051
25-36 months	66	29	43.9	0.277
37-60 months	61	27	44.3	0.273
Total	902	340	37.7	

* P-value calculated using Chi-square test

3.3.3 HAdV distribution by hospital

The proportion of HAdV positive episodes (number of positive HAdV/total number of positive episodes) was highest in Bintulu hospital (42.4%) followed by those from SGH (40.8%) and Sibu hospital (30.9%) as shown in Table 3.17.

Table 3.17: Human adenovirus positivity by hospital

Hospital	Total positive episodes		HAdV positivity	
	n= 902	%	n=340	%
SGH	164	18.2	67	40.8
Sibu Hospital	349	38.7	108	30.9
Bintulu Hospital	389	43.1	165	42.4

3.3.4 HAdV species distribution

All HAdV positive samples were sequenced. Analysis by basic local alignment search tool (BLAST) sequencing (http://blast.ncbi.nlm.nih.gov/Blast.cgi) identified thirteen different serotypes. These included species B (HAdV-3, 7, and 11), species C (HAdV-1, 2, 5, and 6), species D (HAdV-8, 36, 54), species E (HAdV-4) and species F (HAdV-40, 41) as shown in Table 3.18. Species B, mainly serotype 7, was the most common type, accounting for about half of the total positive (51.8%, 176/340), followed by serotype 3 (19.1%, 65/340) and HAdV species C, serotype 2 (11.8%, 40/340).

Table 3.18: Distribution of HAdV molecular types among different species, November 2012-October 2015

Species	**Molecular type**	**No. of detection (%)**
Species B	HAdV-3	65 (19.1)
	HAdV-7	176 (51.8)
	HAdV-11	1 (0.3)
Species C	HAdV-1	21 (6.2)
	HAdV-2	40 (11.8)
	HAdV-5	15 (4.4)
	HAdV-6	10 (2.9)
Species D	HAdV-8	1 (0.3)
	HAdV-36	1 (0.3)
	HAdV-54	1 (0.3)
Species E	HAdV-4	3 (0.9)
Species F	HAdV-40	1 (0.3)
	HAdV-41	5 (1.5)
	Total	**340 (100)**

3.3.4.1 HAdV seasonality

Table 3.19 showed the yearly variation of HAdV detection from three study hospitals. The 340 positive episodes consisted of 202 (59.4%) obtained in 2012-2013, 33 (9.7%) in 2013-2014 and 105 (30.9%) in 2014-2015. Higher detection rates were noted in 2012-2013 and 2014-2015 season. Overall, most of the HAdV was detected between April and August with a peak in the month of July (68.75%). Two peaks (notable rises in the HAdV detection) occurred (Figure 3.17), one in 2012-2013 season and one in 2015.

Table 3.19: Total HAdV distribution by month/year

	1st season (2012-2013)	2nd season (2013-2014)	3rd season (2014-2015)	Whole study period	Total positive episodes	Percentage
Nov	4	3	2	9	51	17.65%
Dec	16	8	0	24	64	37.50%
Jan	14	8	2	24	107	22.43%
Feb	13	6	2	21	95	22.11%
Mar	18	2	5	25	87	28.74%
Apr	27	2	2	31	93	33.33%
May	21	2	6	29	62	46.77%
Jun	22	1	10	33	78	42.31%
Jul	31	1	34	66	96	68.75%
Aug	18	0	17	35	64	54.69%
Sep	13	0	9	22	56	39.29%
Oct	5	0	16	21	49	42.86%
Grand Total	202	33	105	340	902	

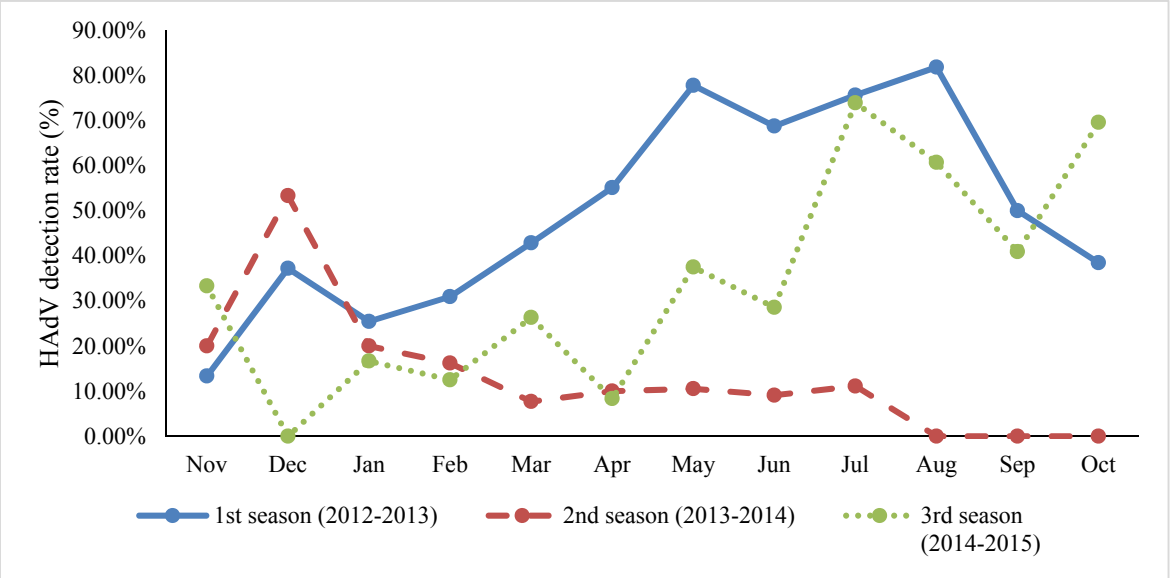


Figure 3.17: Monthly trends of HAdV infection in Sarawak during each of the three consecutive epidemic seasons

We investigated the temporal distribution of HAdV from three study hospitals in Sarawak from November 2012-October 2015. Geographically, the pattern of HAdV distribution was similar throughout the three study hospitals as shown in Figure 3.18. We also observed that HAdV peaks occurred in alternate years in our study.

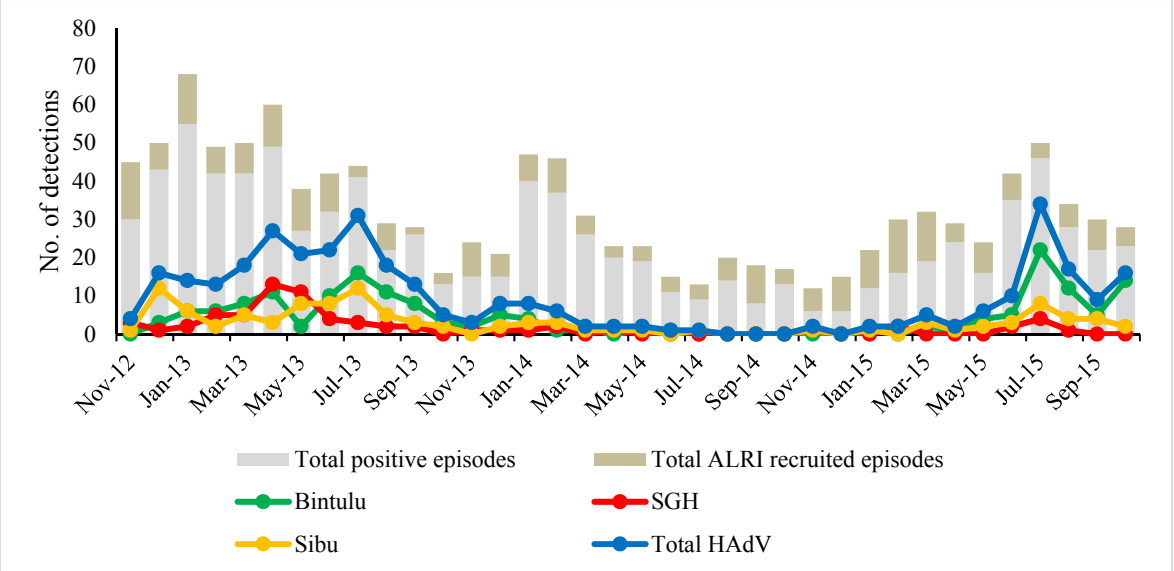


Figure 3.18: Monthly trends of HAdV detection in the three study hospitals

Looking at the distribution of HAdV episodes over time, we observed that increases in HAdV detection in 2012-2013 and 2015 mainly involved HAdV species B (Figure 3.19), whereas HAdV-C and other HAdV species was detected sporadically throughout the study period.

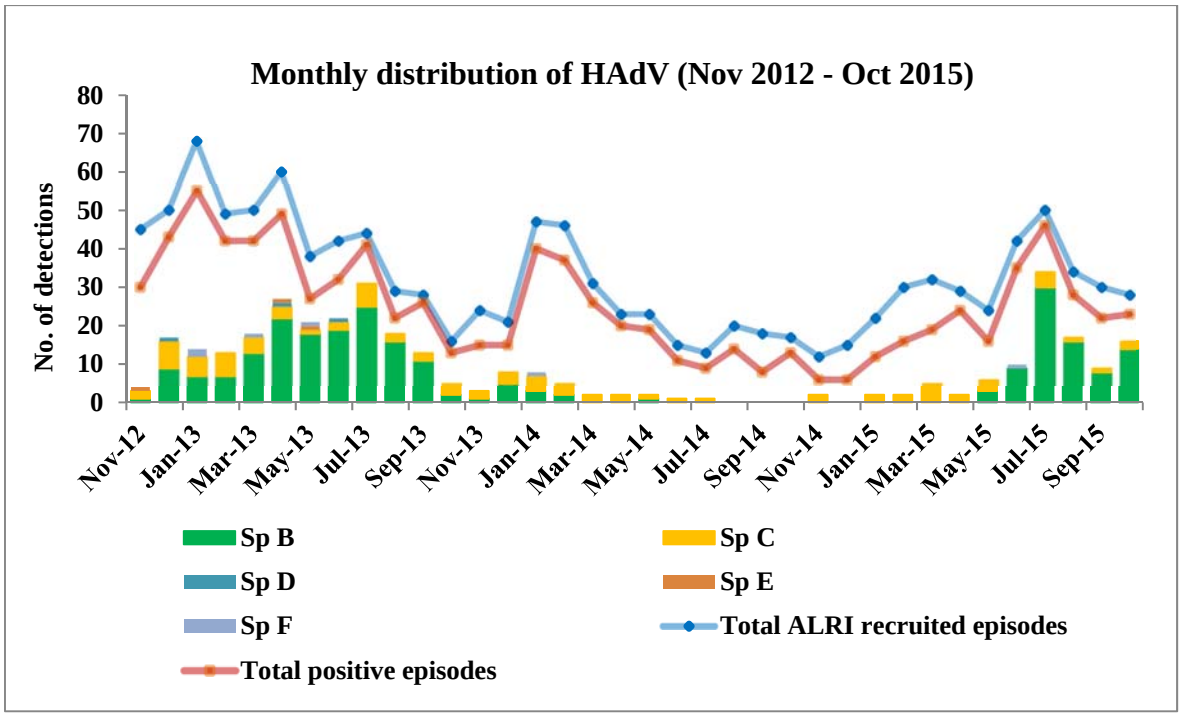


Figure 3.19: Total monthly HAdV species distribution (November 2012-October 2015)

As shown in Figure 3.20, in 2012-2013 seasons, we detected HAdV throughout the year (except November 2012) in Bintulu hospital with an increased HAdV detection between April and August with a peak in July 2013. As for SGH, HAdV was also detected throughout the year except October 2013 with a peak in April and May 2013. On the other hand, Sibu hospital showed a peak in December 2012 and July 2013. This increase was predominantly related to HAdV-7, which was first detected in November 2012 in Sibu Hospital and represented 70.8% (n=143) of the 202 genotyped HAdV detected in the first year of study (Figure 3.21).

In 2013-2014 season, there was a decrease in HAdV detection. In Bintulu hospital, only five HAdV was detected during this period of time, no HAdV was detected in April 2014 and July 2014 to December 2014. For SGH, only 6 positive HAdV were reported, no HAdV was detected in March 2014 and May to October 2014. Similarly, Sibu Hospital also detected only 12 HAdV during the second season of the study, no HAdV was detected in November 2013, June 2014 and August to October 2014.

During the third season (2014-2015), there was an increase in HAdV detection rate towards the end of the study. All three study hospitals showed an increase in HAdV detection in July 2015. Among 105 HAdV detected, Bintulu had the highest detection rate of 63% (66/105). Of the 105 HAdV detected and genotyped during November 2014 to October 2015, 54.2% (n=57) were HAdV-3 while 21.9% (n=23) were HAdV-7 (Figure 3.20). It is interesting to note that there was a shift of serotype from HAdV-7 to HAdV-3 for the two peaks that was observed.

In this study, an association with seasonality was observed for HAdV in which activity peaks during hot months in 2013 and 2015. HAdV was positively correlated with mean temperature ($r= 0.384$, $p < 0.05$) but negatively correlated with rainfall ($r = -0.272$, $p = 0.109$) (Figure 3.22).

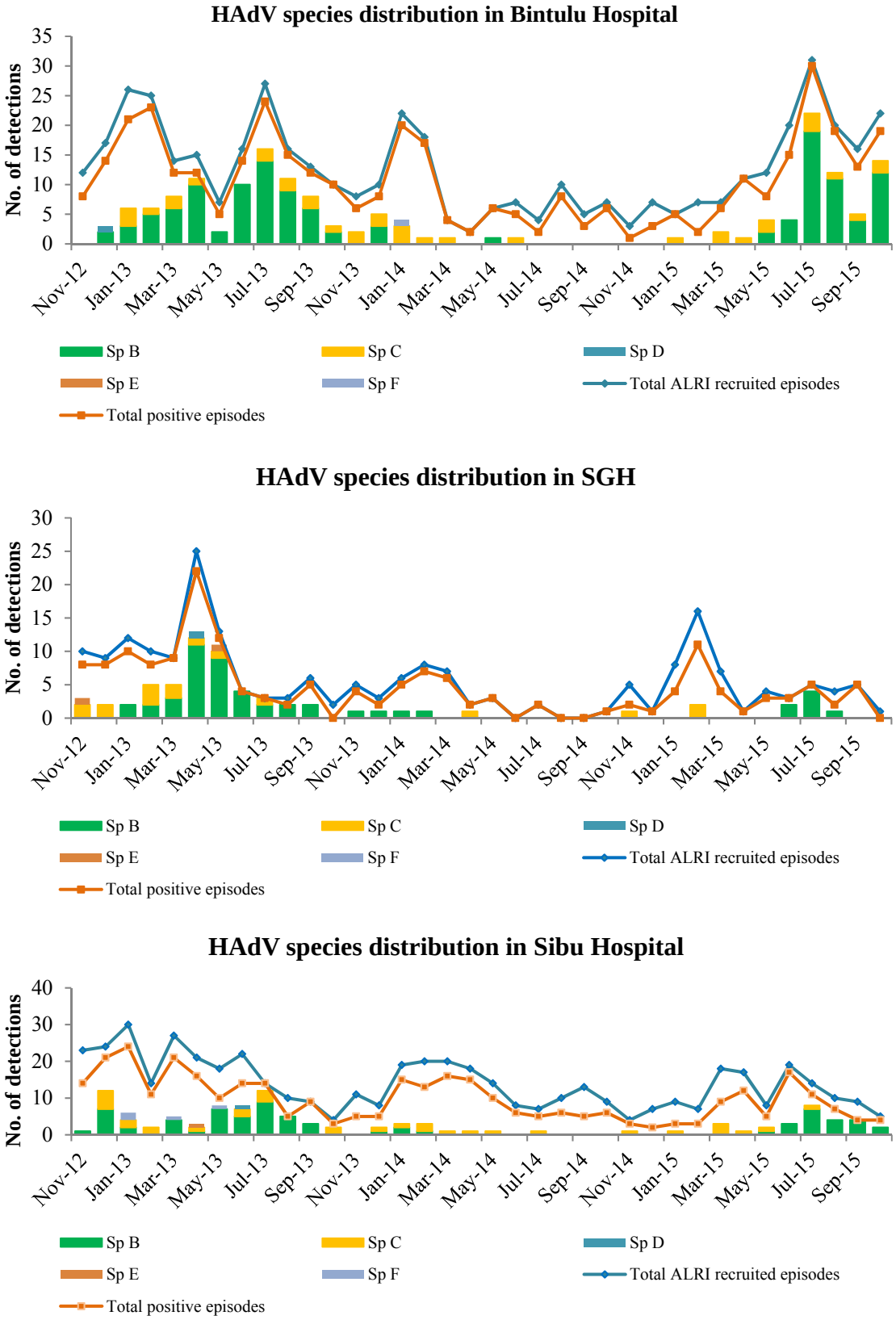


Figure 3.20: Temporal distributions of human adenovirus species in each study hospital in Sarawak, from November 2012 to October 2015

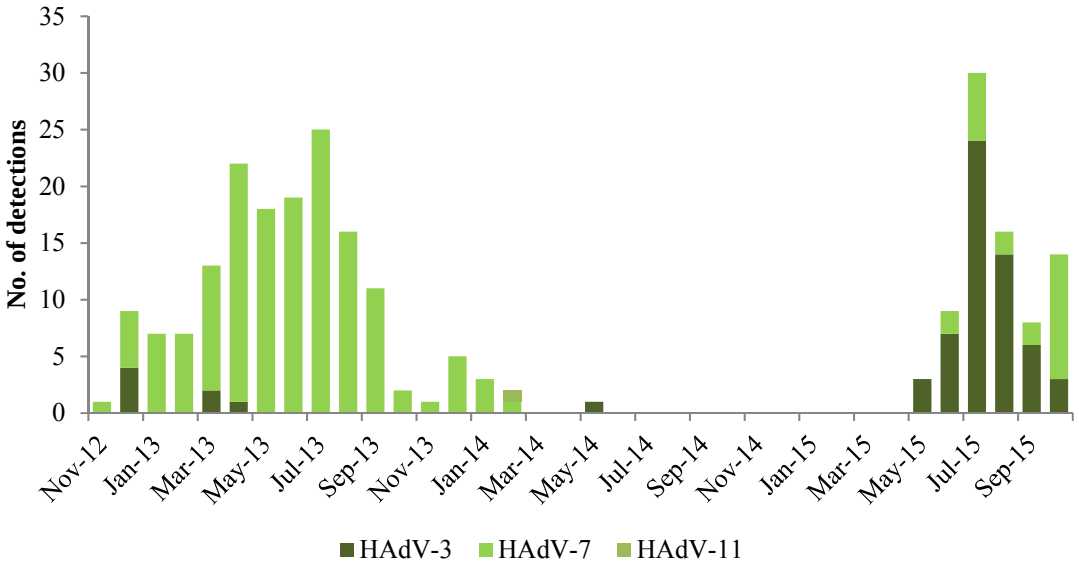


Figure 3.21: Temporal distribution of human adenovirus species B detected between November 2012 and October 2015.

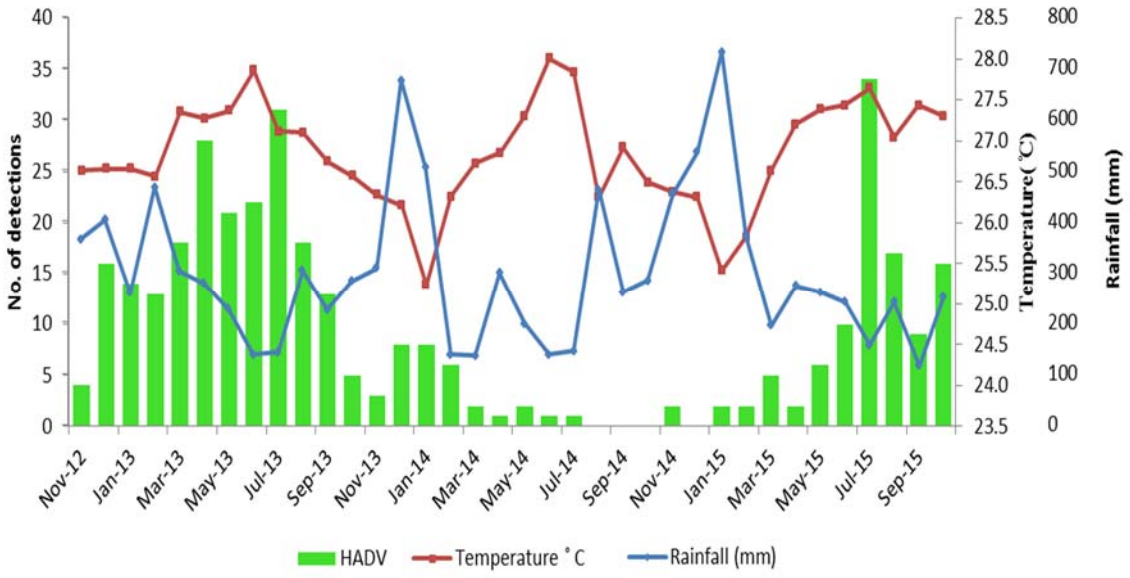


Figure 3.22: HAdV seasonality in association with meteorological factors

3.3.4.2 HAdV phylogenetic analysis

The phylogenetic tree shown in Figure 3.23 includes the representative strains sequenced in this work, along with sequences obtained from GenBank. They were grouped into six clusters, corresponding to HAdV species A, B, C, D, E and F. The hexon gene BLAST analysis was concordant with the HAdV phylogenetic tree clustering. Demographically, HAdV strains in this study were most closely related with strains originated from China, USA, Malaysia and Thailand.

Separate phylogenetic tree was constructed for HAdV species B of HAdV-3 and HAdV-7 (Figure 3.24). The analysed HAdV- 3 hexon gene sequences were found clustered together with previously reported strains from Malaysia and other reference strains from Thailand. As HAdV-7, the hexon gene sequences showed they clustered together with reference sequences from Malaysia, USA and China.

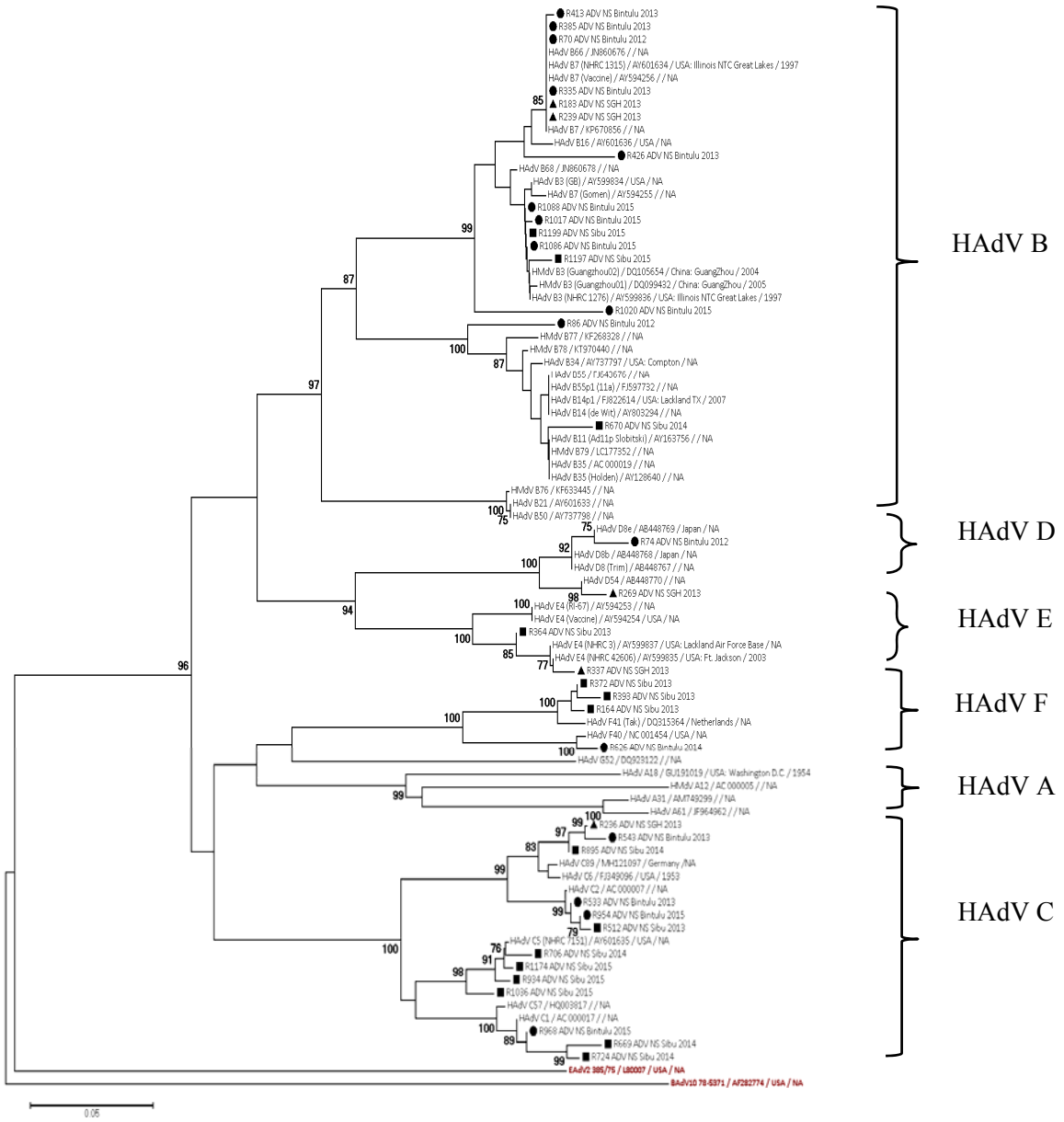


Figure 3.23: Phylogenetic tree of HAdV. Phylogenetic tree of adenovirus hexon gene sequences of representative sequences from our study and other reference strains. Neighbor-joining trees were constructed and the distance was calculated according to Kimura's two-parameter method, with labelling of branches showing at least 70% bootstrap support. The ● indicate samples from Bintulu hospital, ■ indicate samples from Sibu hospital and ▲indicate SGH. Reference strains of HAdV available in GenBank are indicated by type, accession numbers, country and years. Sequences from this study are designated by patient number, the study hospital and year of collection. The outgroups were represented in red.

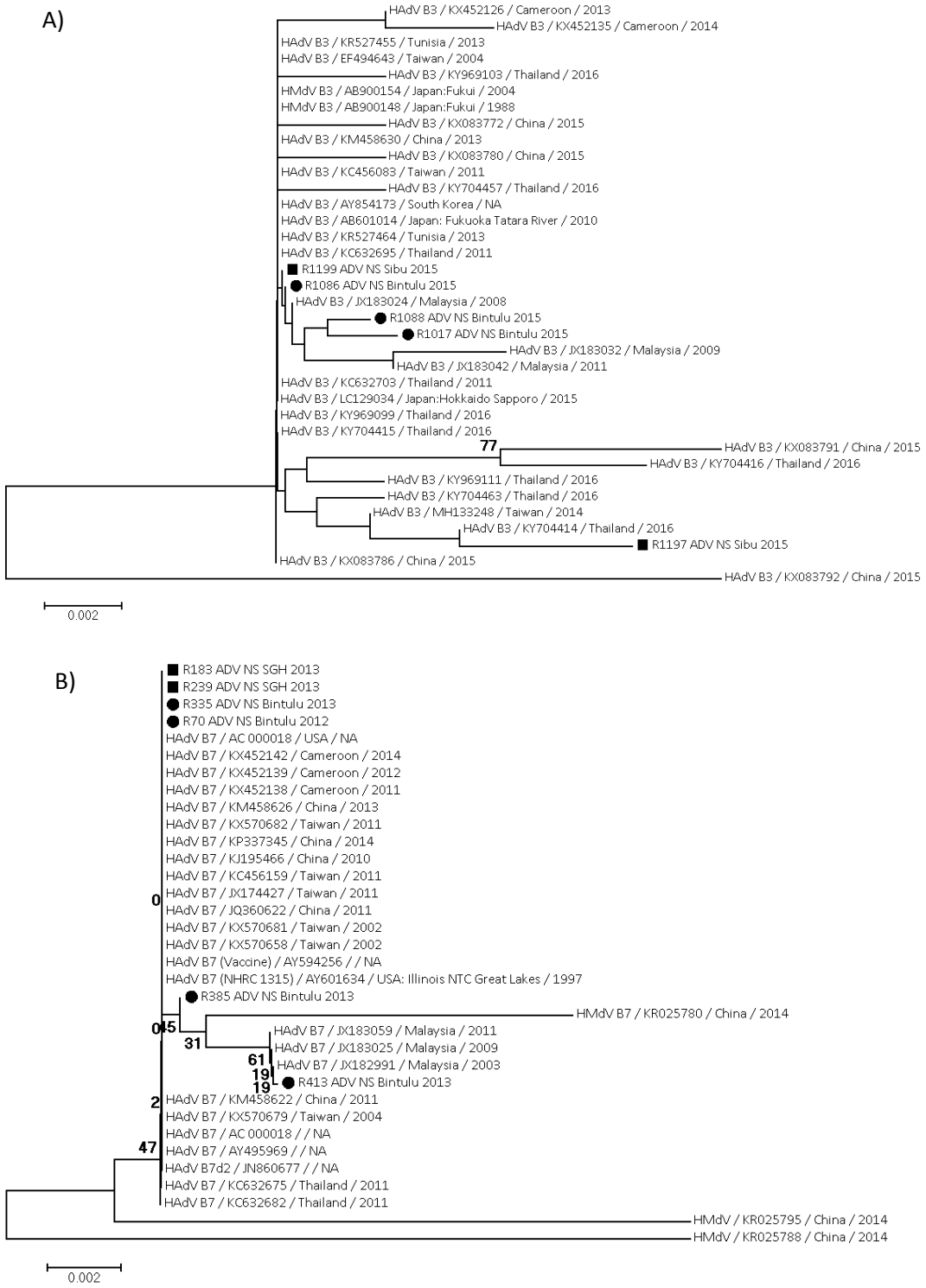


Figure 3.24: Phylogenetic analysis of adenovirus type 3 and type 7 hexon genes. The phylogenetic tree was inferred from HAdV-3 hexon gene (A) and HAdV-7 hexon gene (B). Tree topology was constructed using the neighbour-joining method. Reference strains of HAdV available in GenBank are indicated by type, accession numbers, country and years. Sequences from this study are designated by patient number, the study hospital and year of collection.

3.3.5 HAdV and co-detection

HAdV was identified as a single agent in 41.8% (n=142) of the total positive episodes. It was observed that there was a high co-detection rate between HAdV and other respiratory viruses in our study (Table 3.20). One or more viruses were detected in 58.2% (n=198) of the total HAdV positive episodes. Dual detection was found in 50% (n=170), triple detection in 7.9% (n=27) and one quadruple detection was found among 340 HAdV positive episodes. The most common virus co-detected with HAdV was RSV (26.5%, 90/340) followed by HRV (17.9%, 61/340). Figure 3.25 shows the HAdV co-detection pattern mainly with RSV and other respiratory viruses. As described earlier, most of the HAdV single detection appeared mostly after RSV epidemic season which might indicate that during non-RSV season, HAdV may be the main causative agent involved in ALRI.

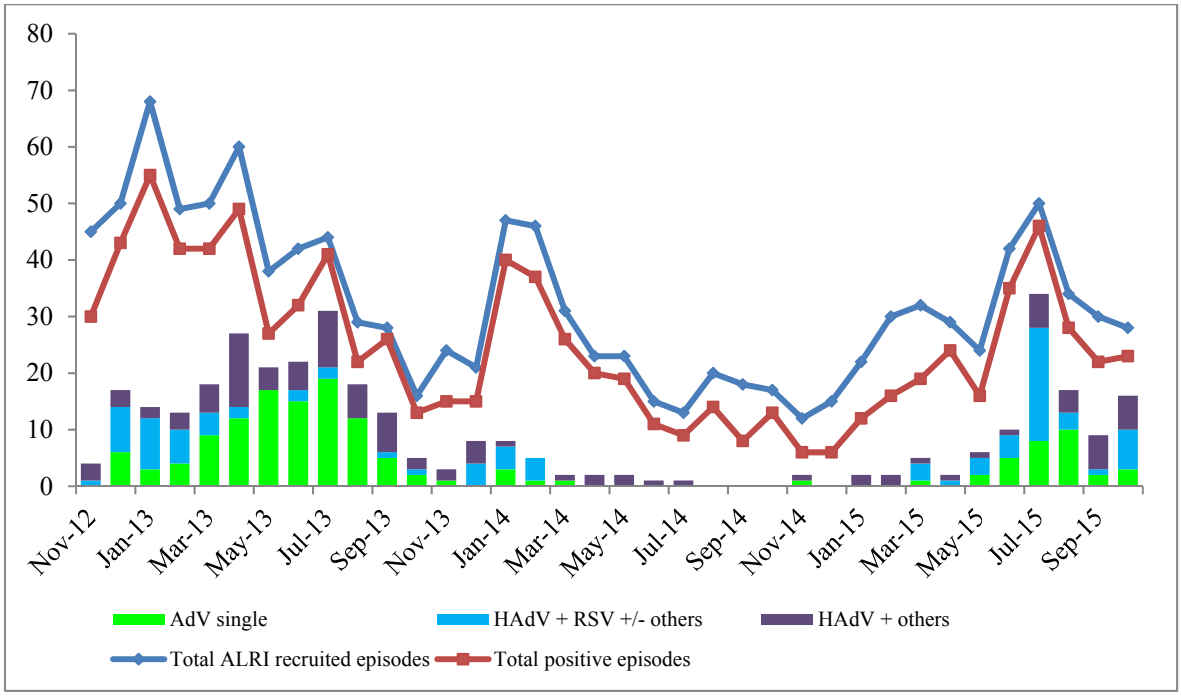


Figure 3.25: Monthly distributions of HAdV and HAdV co-detection

Table 3.20: Distribution of HAdV and co-detection with other respiratory viruses

Respiratory Viruses	Number of Episodes	Percentage (%)
AdV single	142	41.76
AdV Co-detection	198	58.24
HAdV + RSV	73	21.47
HAdV + HRV	46	13.53
HAdV + HPIV	21	6.18
HAdV + HMPV	19	5.59
HAdV + HBoV	5	1.47
HAdV + IFV	4	1.18
HAdV + EV	2	0.59
HAdV + RSV + HPIV	5	1.47
HAdV + RSV + HCoV	2	0.59
HAdV + RSV + IFV	1	0.29
HAdV + RSV + HRV	8	2.35
HAdV + HRV + HPIV	3	0.88
HAdV + HRV + HCoV	2	0.59
HAdV + HRV + HMPV	1	0.29
HAdV + HRV + HBoV	1	0.29
HAdV + HPIV + IFV	1	0.29
HAdV + HMPV + HPIV	1	0.29
HAdV + EV + HBoV	1	0.29
HAdV + EV + HCoV	1	0.29
HAdV + RSV + HBoV + HCoV	1	0.29
Total	340	100.00

3.4 Human rhinovirus (HRV)

3.4.1 HRV distribution by age and gender

Respiratory samples from 1165 paediatric patients admitted to PICU ward were tested for the presence of HRV and other respiratory viruses. A total of 200 (15.5%) children were detected as HRV/EV positive by using multiplex PCR method targeting VP4/VP2/5'-NCR region. To further confirm the subtype of HRV/EV, HRV/EV positive samples from hospitalized patients were further characterized by RT-PCR amplification of a partial VP4/VP2 gene region. Sequencing analysis of the VP4/VP2 region showed that 181 of the 200 HRV/EV multiplex positives were identified as HRV. Other enteroviruses detected will be discussed in following sections. Among the 181 HRV positive episodes detected, 68 (18.6%) were female and 113 (21.1%) were male. The male to female ratio was 1.7:1 (χ^2= 2.05, p= 0.152). As shown in Table 3.21, 122 (22.8%) of the children with HRV detection were aged < 1 year, 37 (15.4%) were aged 13-24 months, 10 (15.2%) were aged 25-36 months and 12 (19.7%) were aged 37- 60 months. The pattern of HRV distribution among hospitals were similar across all age groups with the highest prevalence in children aged < 1 years old.

Table 3.21: Demographic characteristics of patient recruitment

Characteristics	All	HRV Positive [a]	HRV Positivity by hospital		
			SGH	Sibu	Bintulu
Total	n=902 (%)	n=181 (%)	n= 40(%)	n=59(%)	n= 82(%)
Gender					
Female	366(40.6)	68 (18.6)	18 (26.5)	22 (32.4)	28 (41.2)
Male	536 (59.4)	113 (21.1)	22 (19.5)	37 (32.7)	54 (47.8)
Age groups (Month)					
1- 12 months (n= 654)	535 (59.3)	***122 (22.8)[b]***	30 (24.6)	28 (23.0)	64 (52.5)
13-24 months (n=312)	240 (26.6)	37 (15.4)	5 (13.5)	19 (51.4)	13 (35.1)
25-36 months (n=93)	66 (7.3)	10 (15.2)	3 (30.0)	5 (50.0)	2 (20.0)
37-60 months (n=106)	61 (6.8)	12 (19.7)	2(16.7)	7 (58.3)	3 (25.0)

[a] Number (%) of HRV-positive episodes in each characteristics/ total positive episodes of each characteristics

[b] HRV positive episodes by age groups in 1-12 months: $p < 0.05$, calculated using Chi-square test

3.4.2 HRV distribution by hospital

The distribution of HRV among the three study hospitals is depicted in Table 3.22. SGH contributed the highest HRV positive episodes (24.4%) followed by Bintulu hospital (21.1%) and Sibu Hospital (16.9%).

Table 3.22: Human Rhinovirus positivity by hospital

Hospital	Total episodes		HRV positivity	
	n= 902	%	n=181	%
SGH	164	18.1	40	24.4
Sibu Hospital	349	38.7	59	16.9
Bintulu Hospital	389	43.1	82	21.1

3.4.3 HRV seasonality

Looking into the total distribution of HRV based on month, HRV was detected throughout the year. Overall, HRV was detected predominantly in the period between September to November with the highest percentage of 34.69% in the month of October (Table 3.23). However, the numbers fluctuated irregularly during the 3-year study period (Figure 3.23).

We observed that during the first season of study, HRV detection rate was similar throughout ranging between 11% to 30.95% with a highest detection rate found in the month of February. In the second season of study, HRV was detected at a higher rate in Nov-2013 and Oct 2014 while in the third season; most of the HRV was detected between January to February and between September to October. This result is depicted in Table 3.23 and Figure 3.26. The distribution of HRV among the three study hospitals also showed year round HRV detection without a clear seasonal distribution pattern (Figure 3.27).

Table 3.23: Total HRV distribution by month/year

	1st season (2012-2013)	2nd season (2013-2014)	3rd season (2014-2015)	Whole study period	Total positive episodes	Percentage
Nov	6	6	2	14	51	27.45%
Dec	7	3	1	11	64	17.19%
Jan	10	1	5	16	107	14.95%
Feb	13	1	8	22	95	23.16%
Mar	8	3	4	15	87	17.24%
Apr	10	5	5	20	93	21.51%
May	3	3	4	10	62	16.13%
Jun	4	2	1	7	78	8.97%
Jul	9	2	8	19	96	19.79%
Aug	3	2	9	14	64	21.88%
Sep	5	2	9	16	56	28.57%
Oct	2	5	10	17	49	34.69%
Grand Total	80	35	66	14	902	

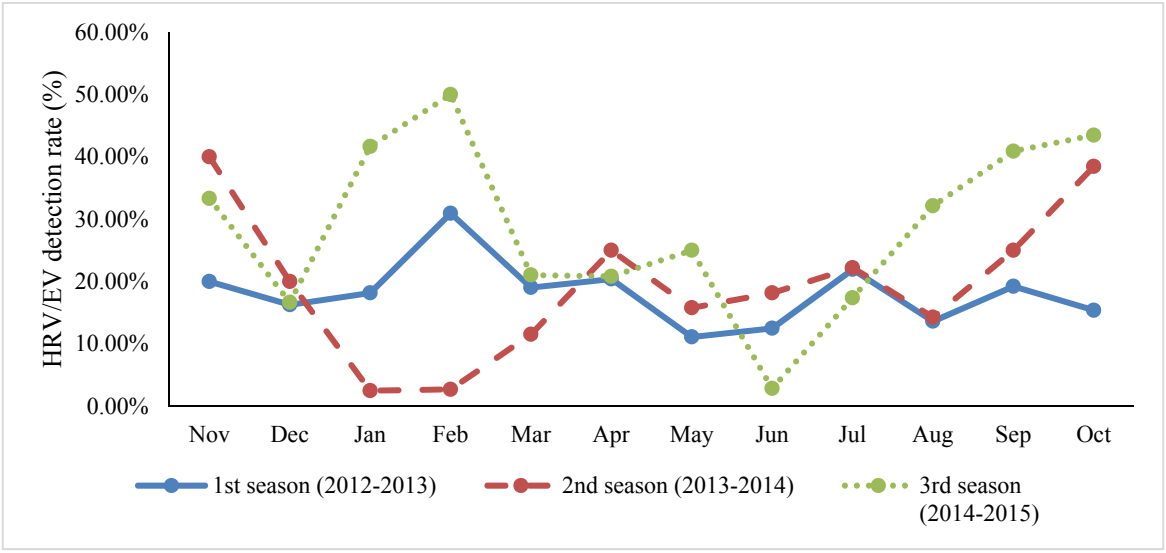


Figure 3.26: Monthly trends of HRV infection in Sarawak during each of the three consecutive epidemic seasons

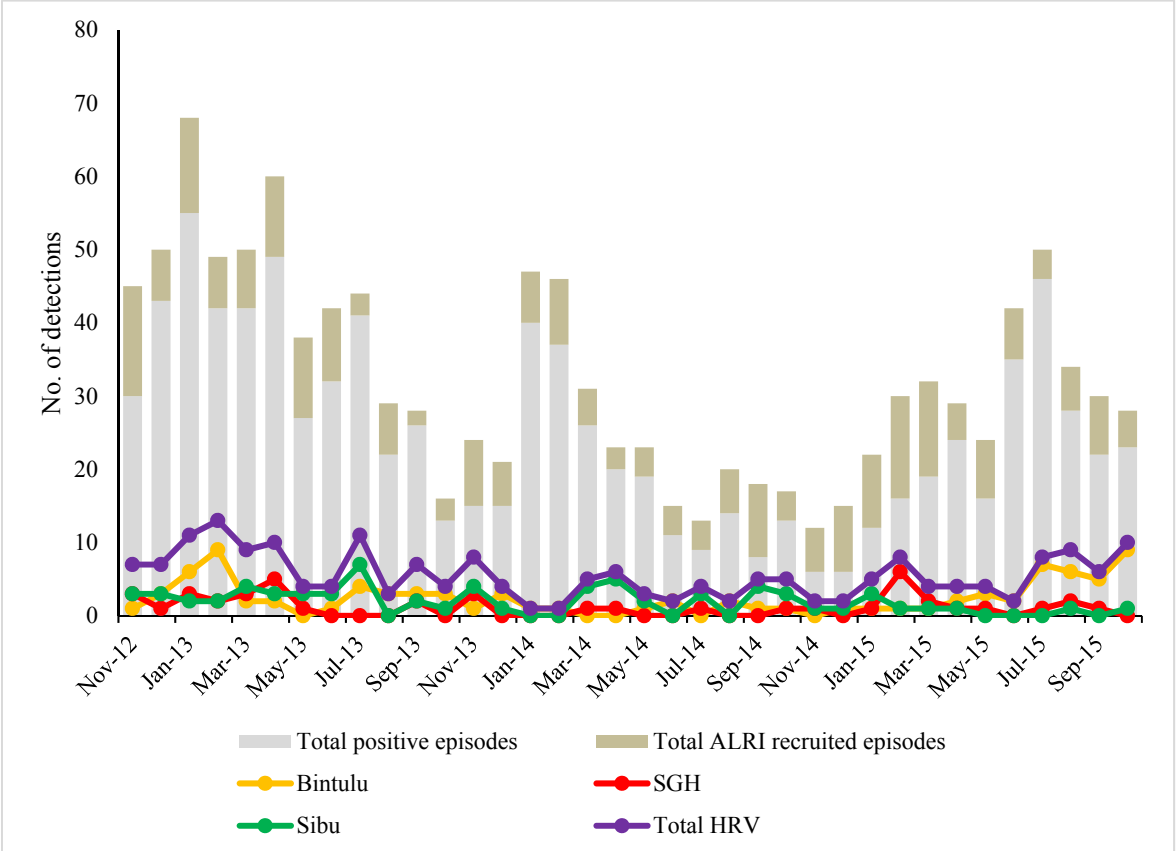


Figure 3.27: Monthly trends of HRV detection in the three study hospitals

3.4.4 HRV and co-detection

HRV was co-detected with at least 1 other virus in 98 of 181 (54.1%) HRV positive episodes. The viruses most frequently co-detected with HRV were adenovirus (HAdV) which accounts for 25.4% (n= 46) followed by respiratory syncytial virus (RSV), 13.3% (n=24) and parainfluenza virus (HPIV), 3.9% (n= 7). Other viruses were co-detected less frequently (Table 3.24). Figure 3.28 shows co-detections of HRV with other respiratory viruses.

Table 3.24: Distribution of RSV and co-detection with other respiratory viruses

Respiratory Viruses	Number of Episodes	Percentage (%)
HRV Single	83	45.90
HRV +HAdV	46	25.4
HRV + RSV	24	13.3
HRV + HPIV	7	3.9
HRV + IFV	2	1.1
HRV + HBoV	2	1.1
HRV + HCoV	1	0.6
HRV +HAdV + RSV	8	4.4
HRV +HAdV + HPIV	3	1.7
HRV +HAdV + HCoV	2	1.1
HRV +HAdV + HMPV	1	0.6
HRV +HAdV + HBoV	1	0.6
HRV + RSV + HPIV + HCoV	1	0.6
Total	**181**	**100**

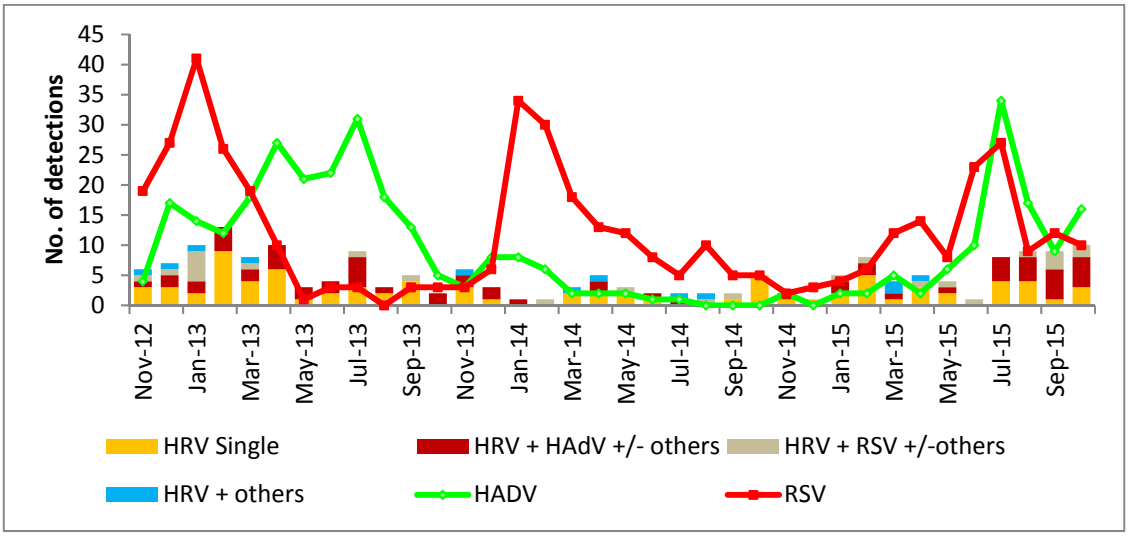


Figure 3.28: Monthly distributions of HRV and HRV co-detection

3.4.5 HRV antigenic group

All 181 HRV positive samples were typed using the VP4/VP2 method. By using this method, 48.6% (88/181) could be typed as HRV-A, 3.9% (7/181) as HRV-B, 47.5% (86/181) as HRV-C. Generally, HRV-A, HRV-B and HRV-C were predominantly found in patients aged < 1 year as shown in Figure 3.29. HRV species was significantly distributed across age group (χ^2 = 19.2, p ≤ 0.01). HRV-C was detected more among those aged 13-24 months which accounted for 67.6% (n= 25) positive episodes whereas HRV-A was detected more among those aged 1-12 months (56.6% positive episodes). HRV-B was the least frequently detected species in all four age categories (Table 3.25).

As shown in Table 3.25, the most common clinical diagnosis of hospitalized children positive for HRV was pneumonia which was exhibited in more than 50% of the total HRV positive episodes. Comparison of clinical diagnosis among HRV species did not show significant difference (χ^2 = 6.331, p= 0.758) between them. Although not statistically significant, it was observed that more HRV-A was detected in pneumonia cases compared to HRV C (50.4% *vs* 45.9%). In addition, it was more likely to detect HRV-C than HRV-A in children with acute exacerbation of bronchial asthma (62.5% *vs* 37.5%). Species distribution did not differ significantly (χ^2 = 5.64, p= 0.215) in the three study hospitals with HRV-A and HRV-C being the predominant species (Table 3.26).

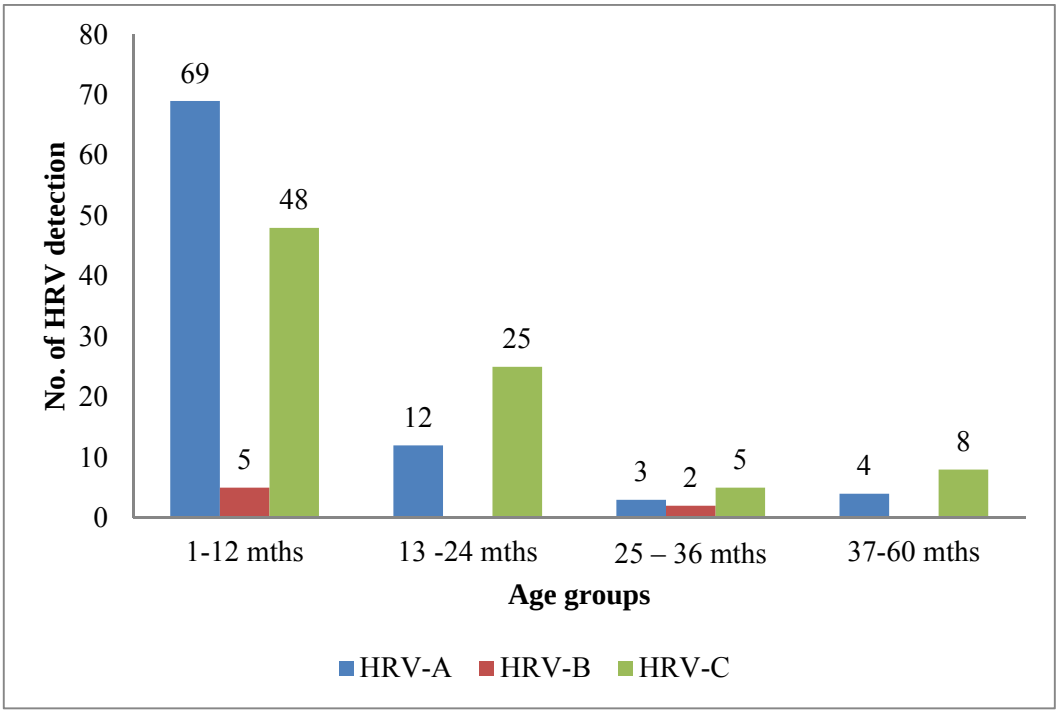


Figure 3.29: HRV species distribution by age group

Table 3.25: Patient's distribution by HRV species

Characteristics	HRV positive episodes n = 181	HRV species n (%)[a]		
		HRV-A *n= 88*	HRV-B *n = 7*	HRV-C *n= 86*
Age group				
1-12 mths	122 (67.4)	69 (56.6)	5 (4.1)	48 (39.3)[b]
13 -24 mths	37 (20.4)	12 (32.4)	0	25 (67.6)[c]
25 – 36 mths	10 (5.5)	3 (30)	2 (20)	5 (50)
37-60 mths	12 (6.6)	4 (33)	0	8 (67.7)
Clinical diagnosis				
Pneumonia	133 (73.5)	67 (50.4)	5 (3.8)	61(45.9)
Acute bronchiolitis	18 (9.9)	9 (50)	2 (11.1)	7 (38.9)
Acute exacerbation of bronchial asthma	16 (8.8)	6 (37.5)	0	10 (62.5)
Newly diagnosed asthma with concurrent respiratory tract infection	8 (4.4)	4 (50)	0	4 (50)
Acute exacerbation of chronic lung disease	2 (1.1)	1(50)	0	1 (50)
Viral wheeze	4 (2.2)	1 (25)	0	3 (75)
Viral croup	0 (0)	0	0	0

[a] Number (%) of HRV-positive episodes in each age group/ clinical diagnosis

[b] HRV A versus HRV-C in age group 1-12 months: $p < 0.05$, calculated using Fisher's Exact test

[c] HRV A versus HRV-C in age group 13-24 months: $p < 0.05$, calculated using Fisher's Exact test

No comparisons were made for HRV-B because of small numbers.

Table 3.26: HRV species predominance by hospital

Hospitals	HRV-A	HRV-B	HRV-C	Total
Bintulu Hospital	45 (54.9%)	4 (4.9%)	33 (40.2%)	82
SGH	20 (50%)	0	20 (50%)	40
Sibu Hospital	23 (39%)	3 (5.1%)	33 (56%)	59

The distribution of HRV species over the 3-year study period is shown in Figure 3.30. HRV-A and HRV-C co-circulated in almost all of the months of surveillance and was rarely absent (e.g., September – November 2014, May 2015 for HRV-A; January-February 2014, June, December 2014 and June 2015 for HRV-C). There is no clear seasonal pattern observed for both HRV-A and HRV-C. In contrast, HRV-B was rarely detected and occurred sporadically throughout the study period. The HRV species distribution among three hospitals is shown in Figure 3.31. There was no clear association between the monthly number of HRV detection and monthly mean surface air temperature ($r^2 = 0.056$, $p = 0.747$) or monthly mean rainfall ($r^2 = -0.154$, $p=0.369$) over the period of study (Figure 3.32).

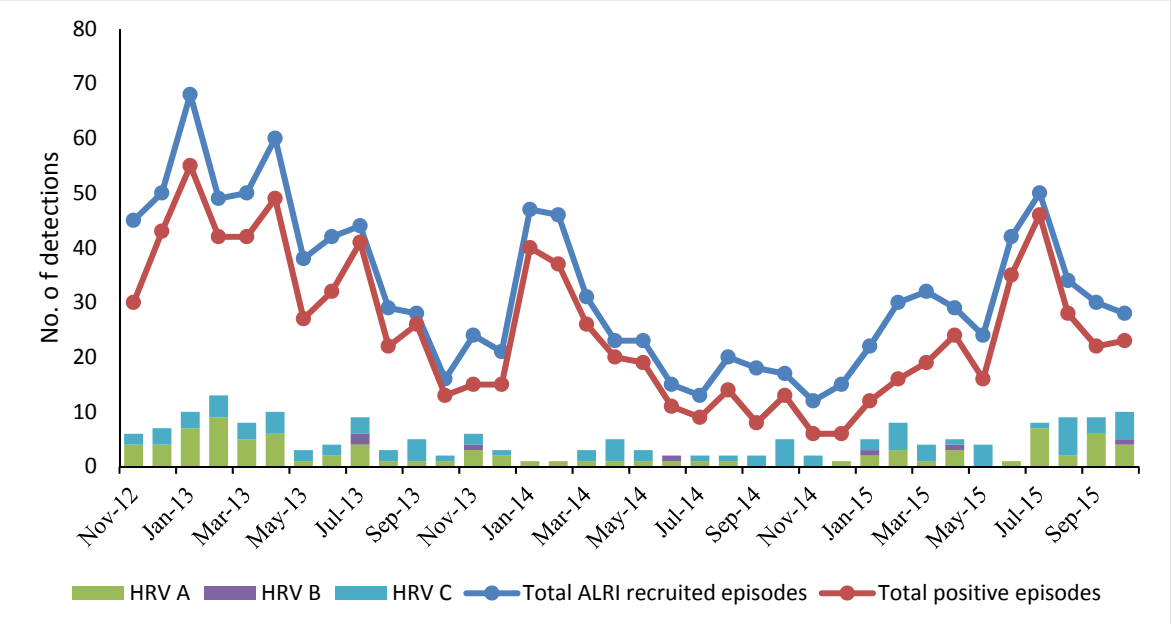


Figure 3.30: Total monthly HRV species distribution (Nov 2012- Oct 2015)

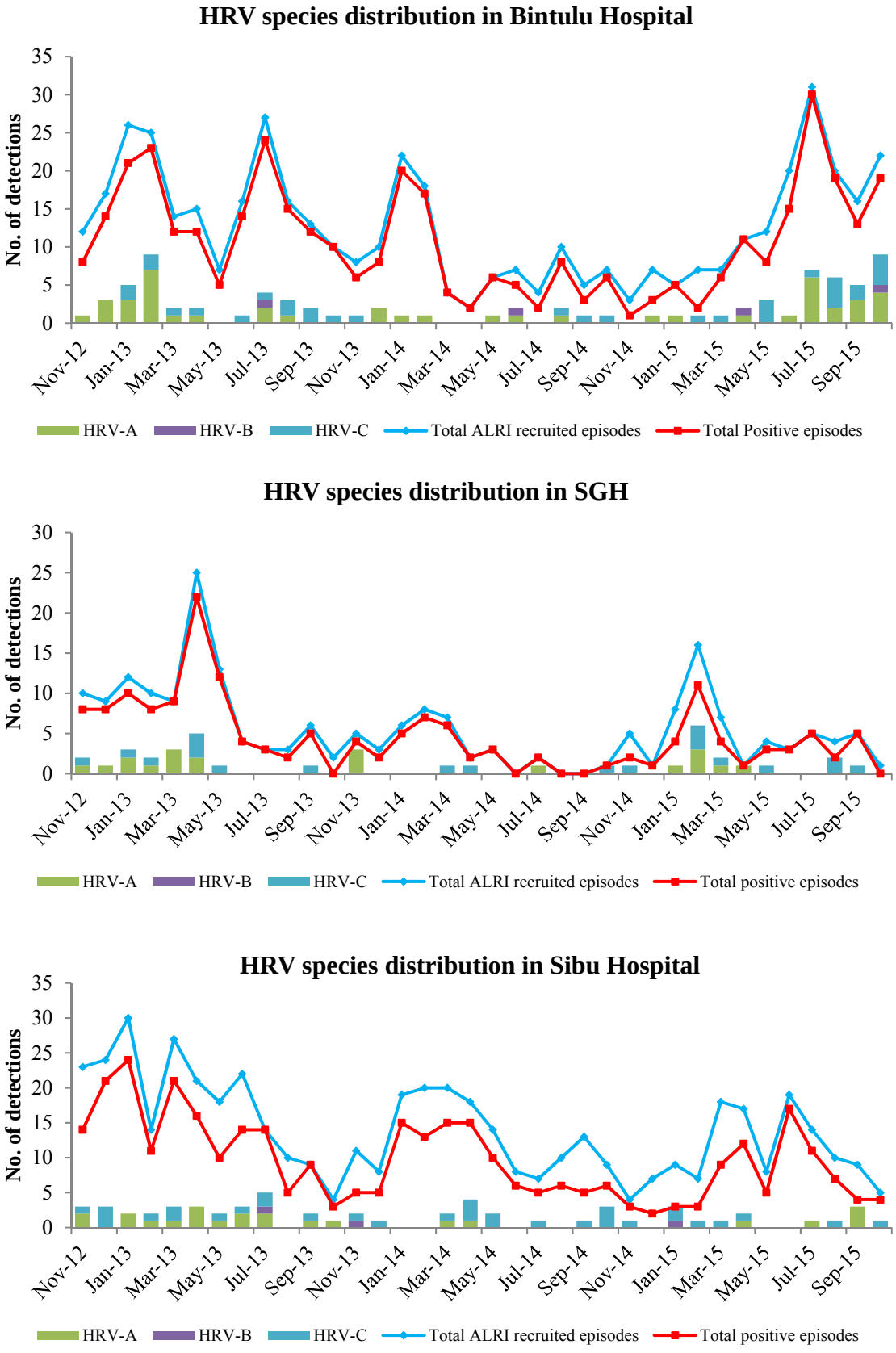


Figure 3.31: Monthly HRV species distribution among three hospitals (Nov 2012 – Oct 2015)

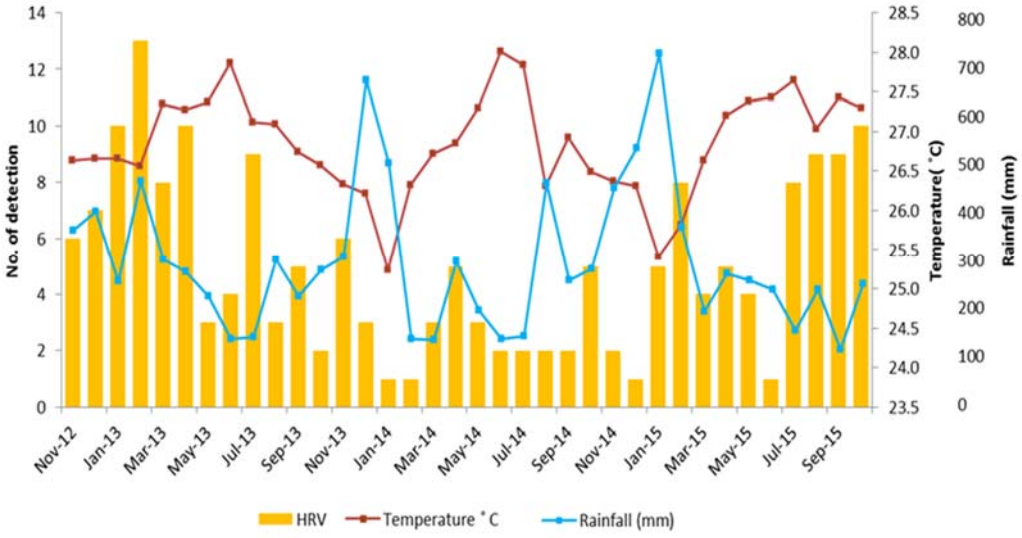


Figure 3.32: HRV seasonality in association with meteorological factors

3.4.5.1 HRV phylogenetic analysis

The VP4/VP2 coding region was RT-PCR amplified and sequenced for the 181 HRV positive samples detected during this study in order to identify the HRV species. Of these, sequence data was obtained for 132 (72.9%) HRV samples with the remaining 49 (27.1%) excluded due to poor sequence quality. Phylogenetic analysis was carried out on all sequences and compared with reference sequences of the respective HRV species obtained from Genbank.

Separate phylogenetic trees were constructed in order to evaluate the genetic diversity within each of the HRV species A, B and C. Viruses were differentiated into genotypically assigned types based on the criteria for HRV species classification presented by Mclntyre *et al.* (2013). A divergence threshold of 10.5, 9.5 and 10.5% was proposed for identifying different HRV-A, B and C types respectively. Based on these criteria, different

circulating HRV types were determined. The 64 HRV-A positive episodes from this study were grouped into 38 types of HRV-A (Figure 3.33), 2 types of HRV-B were detected from 3 HRV-B positive episodes (Figure 3.34), and 37 types of HRV-C were identified from 65 HRV-C positive episodes (Figure 3.35).

Seven variants of potential novel HRV types were identified among HRV-A in which they demonstrated a divergence above the VP4/VP2 nucleotide pairwise distance thresholds proposed for their species. They were therefore designated into provisionally assigned types (PATs) and temporarily named HRV_Apat_a (3 viruses), HRV_Apat_b (1 virus), HRV_Apat_c (1 virus) HRV_Apat_d (1 virus), HRV_Apat_e (2 viruses), HRV_Apat_f (1 virus) and HRV_Apat_g (2 viruses). These viruses await sequence data from their VP1 gene to confirm their assignment as putative new types (Figure 3.33).

Table 3.27 shows the frequencies of HRV genotypes detected among the three hospitals during the study period. Among HRV-A, HRV-A12 was the predominant type (n= 9), followed by HRV-A39 (n= 4), HRV-A21 (n=3) and HRV-A61 (n=3). Eleven HRV-A sequences demonstrated a nucleotide divergence > 10.5% with 81 HRV-A VP4/VP2 gene sequences available in GenBank (Table 3.27). Of these, R49_Sibu_Nov12, R92_Bintulu_Jan13 and R136_Bintulu_Jan13 sequences showed more than 10.5% nucleotide divergence (12.7%, 13.2% and 25.9% respectively) to the most closely related reference sequence (HRV-A22). These three sequences temporarily grouped with the provisionally assigned types designated HRV_Apat_a. R79_Bintulu_Dec12 showed a nucleotide divergence of 11.2% to the closest reference sequence (HRV-A32) and was assigned as HRV_Apat_b. On the other hand, R80_SGH_Dec12 and R924_SGH_Mar15 showed a nucleotide divergence of 11.9% and 16.1% with HRV-A66 and HRV-A12 respectively and thus were assigned as HRV-Apat_c and HRV-Apat_d. R91_Bintulu_Jan13

clustered with HRV-A36 and HRV-A58 which showed a nucleotide divergence of 14.6% and 15.6% respectively and was assigned as HRV-Apat_f. In addition, a series of HRV-A sequences showed a nucleotide divergence > 10.5% from their reference sequences: R75_Bintulu_Dec12 and R1020_Bintulu_Jul15 vs HRV-A88 (11.6% and 11.9%) and R33_Bintulu_Nov12 and R202_Bintulu_Feb13 vs A89 (12.1% and 11.5%). They were assigned as HRV-Apat_e and HRV-Apat_g respectively. A separate study on HRV being conducted at the institute will include VP1 sequencing to confirm type assignment.

The nine strains of HRV-A12 detected in this study circulated in two different seasons (Jan-Apr 2013 and August 2015) in Bintulu Hospital and SGH. Based on the phylogenetic tree on HRV subtypes, the same strain of HRV-A 12 was also found in USA in 2007 and France in 2010 while the four strains of HRV-A39 circulated in 2013 was found to be similar with the strain from Sweden in 2012 (Figure 3.36). The three strains of HRV-A61 circulated in June-July 2013 mainly in Sibu whereas the other three strains of HRV-A21 was found circulating mainly in Bintulu for three consecutive years (2012- 2014) (Figure 3.33). HRV A-21 and HRV-A61 were similar to the strain from China as shown in Figure 3.36.

Table 3.27: HRV provisionally assigned type in this study based on partial VP4/VP2 gene sequences

Patient ID	Nearest HRV Genotype based on VP4/VP2	Divergence with the nearest HRV (% nt)	Homology value (%)	Provisionally assigned type
R49_Sibu	HRV-A22	12.7	88.1	HRV_Apat_a
R92_Bintulu	HRV-A22	13.2	87.8	HRV_Apat_a
R136_Bintulu	HRV-A22	25.9	88.1	HRV_Apat_a
R79_Bintulu	HRV-A32	11.2	89.9	HRV_Apat_b
R80_SGH	HRV-A66	11.9	89.1	HRV_Apat_c
R924_SGH	HRV-A12	16.1	85.6	HRV_Apat_d
R91_Bintulu	HRV –A36/58	14.6/15.6	86.8/85.8	HRV_Apat_f
R75_Bintulu	HRV-A88	11.6	89.4	HRV_Apat_e
R1020_Bintulu	HRV-A88	11.9	89.1	HRV_Apat_e
R33_Bintulu	HRV-A89	12.1	89.1	HRV_Apat_g
R202_Bintulu	HRV-A89	11.5	89.6	HRV_Apat_g

All sequences of HRV-B and HRV-C clustered with known molecular types of HRV around the world based on sequencing of the VP4/VP2 gene (Figure 3.34 & Figure 3.35). The two types of HRV-B circulated include HRV-B69 (n=2) and HRV-B27 (n=1). HRV-B69 was similar to strain from Nepal/Netherlands while HRV-B27 was similar to strain found in China (Figure 3.36).

Recently a proposal for the designation of HRV-C provisional assigned types (PATs) has been published based only on the VP4/VP2 sequences. Following this classification, the 14 PATs together with 53 HRV-C available sequences were used to construct the phylogenetic tree with the 65 HRV-C positive sequences identified in our study. The 65 positive sequences obtained in our study were genetically assigned to 37 types in the phylogenetic tree. As shown in Table 3.28, the most frequent were HRV-Cpat10 (n=7) which was now classified as HRV C-53, HRV-C35 (n=5) and HRV-C11 (n=4). It was observed that there was rapid turnover of the HRV types. The 7 HRV-Cpat10 identified in this study were found to circulate in the 3 study hospitals between December 2012 and April 2013.

This HRV-Cpat10 (HRV-C53) was closely related to the strain found in China in 2014. The other 5 HRV-C35 identified was also found to circulate in the three study hospitals mainly in 2013 except one which was detected in October 2015. These HRV-C35 was closely related to the strain found in Hong Kong in 2008. The four strains of HRV-C11 was found mainly in Bintulu which was first detected in October 2013 and reappeared in July to August 2015. Besides that, they are similar to the strain found in Thailand in 2011& 2012 (Figure 3.36).

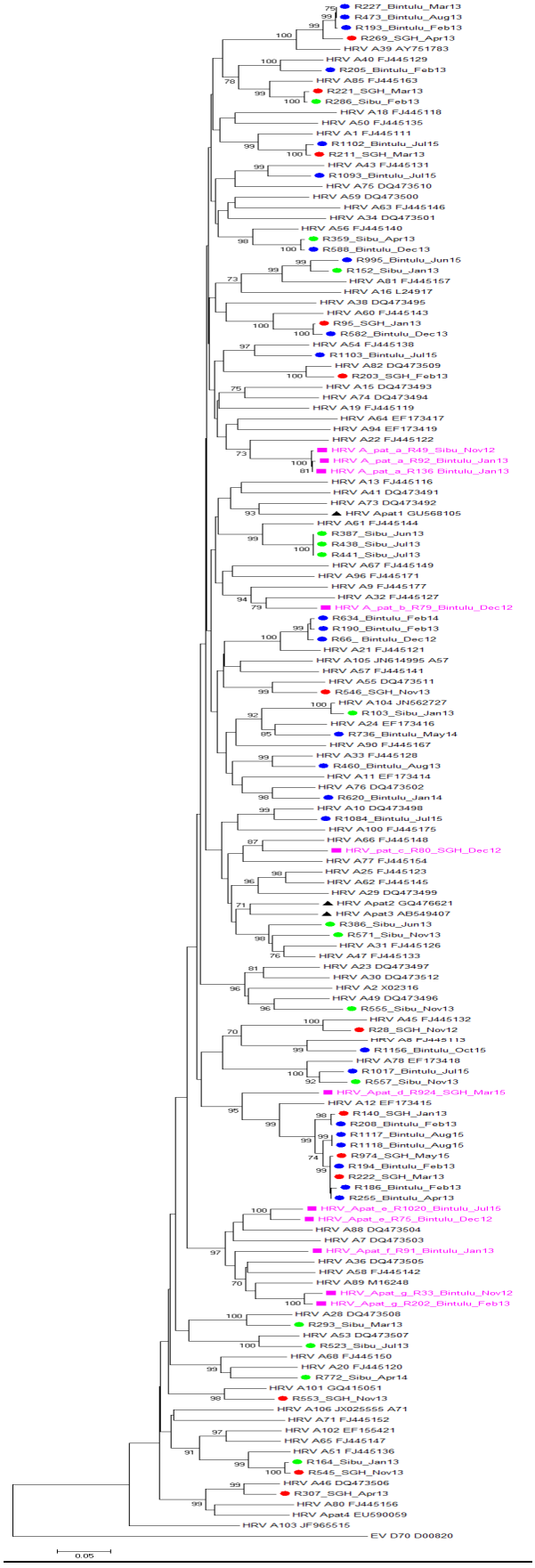


Figure 3.33: Phylogenetic analysis of the VP4/VP2 region of HRV-A. The phylogenetic tree is based on the nucleotide sequence of the VP4/VP2 coding region (394 nt) of the 64 sequences analysed in this study and 77 reference strains representative of each HRV type except HRV A107-109 which are currently not available in GenBank. All phylogenetic analysis was undertaken using the Enterovirus D sequence (GenBank accession no. D00820 for EV-D70) as an outgroup. Neighbor-joining trees were constructed and the distance was calculated according to Kimura's two-parameter method, with labelling of branches showing at least 70% bootstrap support. The coloured dots indicate the sampling site: blue, Bintulu Hospital; green, Sibu Hospital; red, SGH; Black triangles, provisionally assigned types (Pats) of HRV reference strains. Sequences of HRV provisionally assigned types (Pats) from this study are indicated by ■. Reference strains of species A available in GenBank are indicated by their accession numbers. Sequences from this study are designated by patient number, the study hospital, month and year of collection.

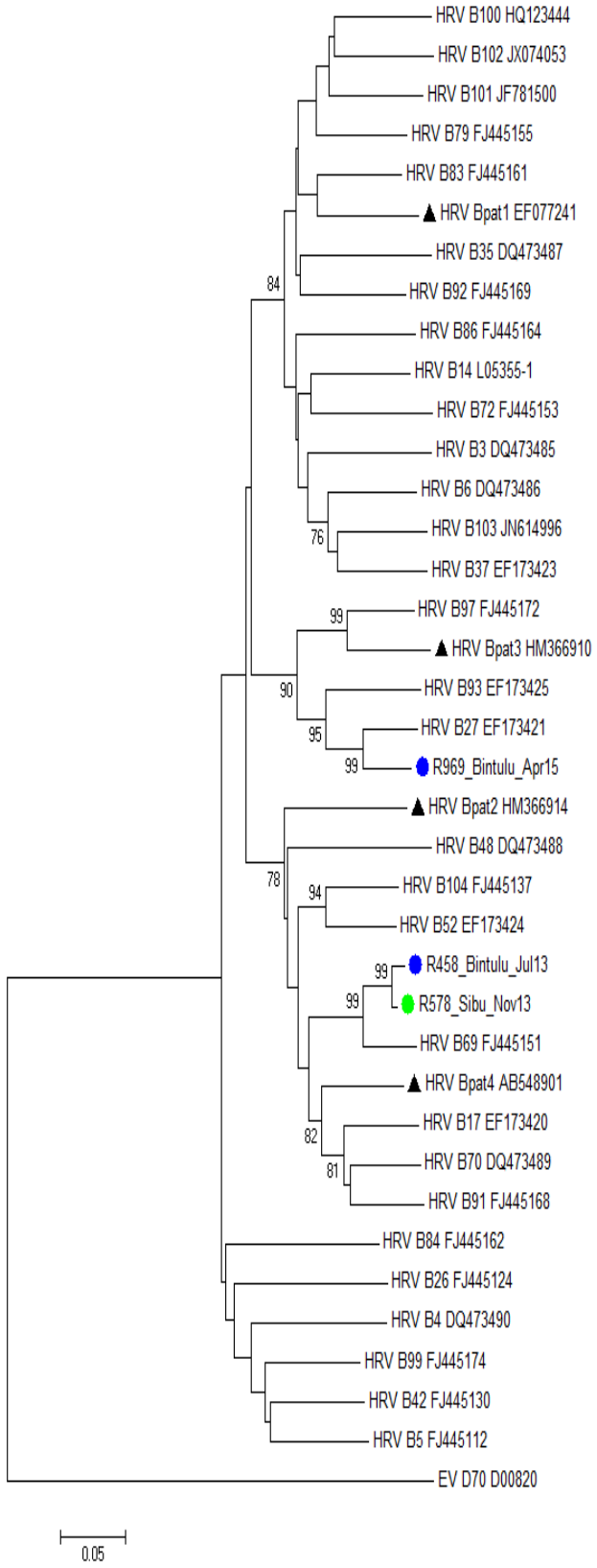


Figure 3.34: Phylogenetic analysis of the VP4/VP2 region of HRV-B. The phylogenetic tree is based on the nucleotide sequence of the VP4/VP2 coding region (394 nt) of the 3 sequences analysed in this study and 32 reference strains representative of each HRV type which are available in GenBank. All phylogenetic analysis was undertaken using the Enterovirus D sequence (GenBank accession no. D00820 for EV-D70) as an outgroup. Neighbor-joining trees were constructed and the distance was calculated according to Kimura's two-parameter method, with labelling of branches showing at least 70% bootstrap support. The coloured dots indicate the sampling site: blue, Bintulu Hospital; green, Sibu Hospital; red, SGH; Black triangles, provisionally assigned types (Pats) of HRV reference strains. Reference strains of species B available in GenBank are indicated by their accession numbers. Sequences from this study are designated by patient number, the study hospital, month and year of collection.

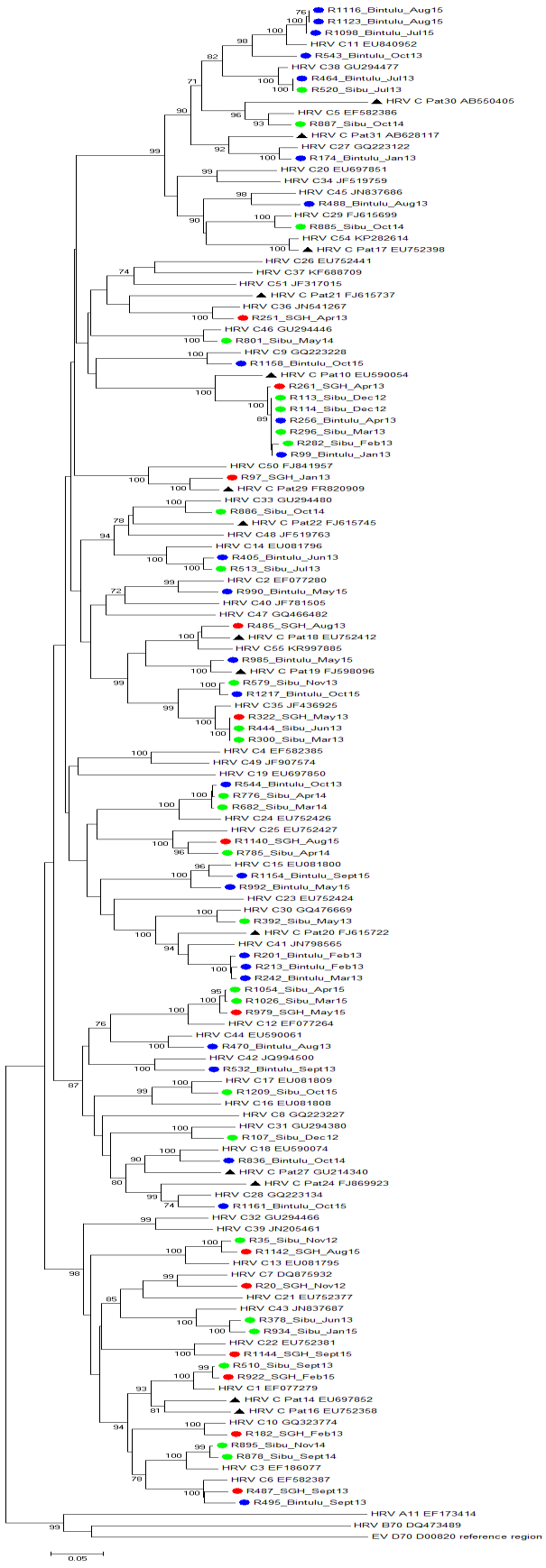
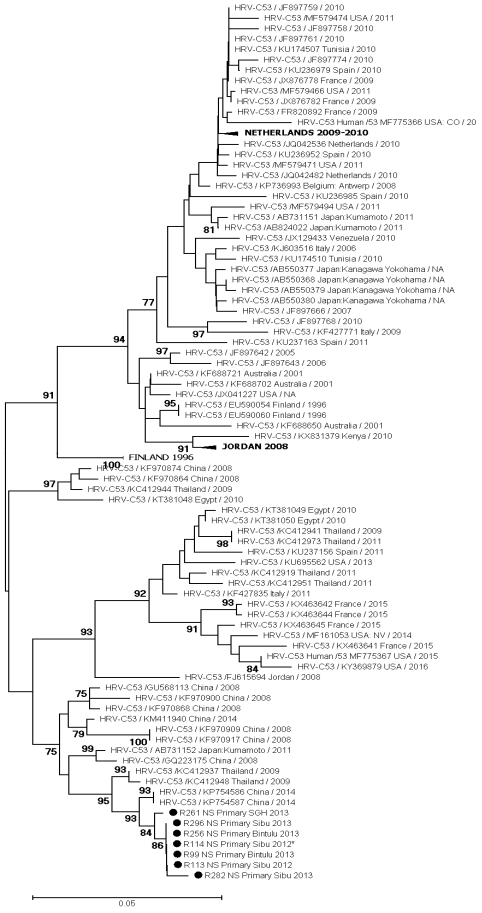


Figure 3.35: Phylogenetic analysis of the VP4/VP2 region of HRV-C. The phylogenetic tree is based on the nucleotide sequence of the VP4/VP2 coding region (388 nt) of the 65 sequences analysed in this study and 67 reference strains representative of each HRV type representative of each HRV type except HRV-C52 and HRV-C53 which are currently not available in GenBank. All phylogenetic analysis was undertaken using the Enterovirus D sequence (GenBank accession no. D00820 for EV-D70) as an outgroup. Neighbor-joining trees were constructed and the distance was calculated according to Kimura's two-parameter method, with labelling of branches showing at least 70% bootstrap support. The coloured dots indicate the sampling site: blue, Bintulu Hospital; green, Sibu Hospital; red, SGH; Black triangles, provisionally assigned types (Pats) of HRV reference strains. Reference strains of species C available in GenBank are indicated by their accession numbers. Sequences from this study are designated by patient number, the study hospital, month and year of collection.



Figure 3.36: Phylogenetic tree on HRV subtype. The phylogenetic tree is based on neighbour-joining analysis including representative HRV-A-12 (A) HRV-A21 (B) HRV-A61 (C) HRV-A39 (D) HRV-B27 (E) HRV-B69 (F) sequences from this study in comparison with the most closely related sequences found in Genbank.

G) HRV C-pat 10 (HRV C-53)



H) HRV C-11

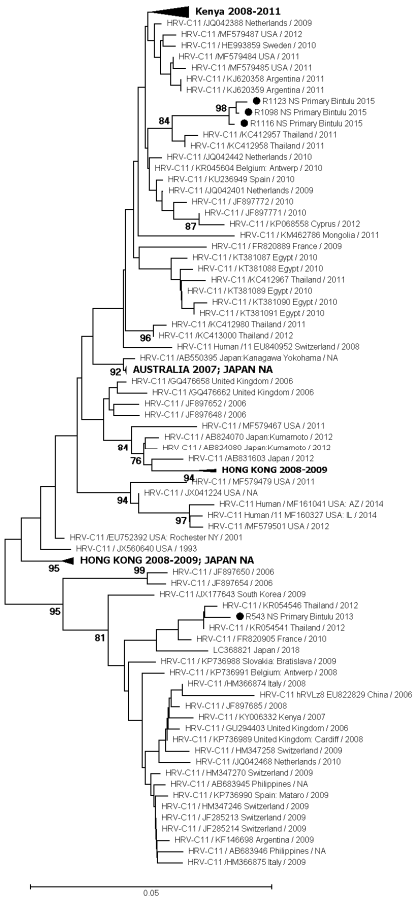


Figure 3.36 continued**:** Phylogenetic tree on HRV subtype. The phylogenetic tree is based on neighbour-joining analysis including representative HRV-C-pat 10 (G) and HRV-C11 (H) sequences from this study, in comparison with the most closely related sequences found in Genbank.

Table 3.28: HRV genotyping based on partial VP4/VP2 gene sequences

VP4/VP2 molecular typing				
Species and type	**Bintulu**	**SGH**	**Sibu**	**Grand Total**
HRV-A (n=38 types)				
HRV A12	6	3		9
HRV A39	3	1		4
HRV A21	3			3
HRV A61			3	3
HRV A1	1	1		2
HRV A51		1	1	2
HRV A56	1		1	2
HRV A60	1	1		2
HRV A78	1		1	2
HRV A81	1		1	2
HRV A85		1	1	2
HRV A8	1			1
HRV A10	1			1
HRV A20			1	1
HRV A24	1			1
HRV A28			1	1
HRV A31			1	1
HRV A33	1			1
HRV A40	1			1
HRV A43	1			1
HRV A45		1		1
HRV A46		1		1
HRV A47			1	1
HRV A49			1	1
HRV A53			1	1
HRV A54	1			1
HRV A55		1		1
HRV A76	1			1
HRV A82		1		1
HRV A101		1		1
HRV A104			1	1
HRV A_pat_a	2		1	3
HRV A_pat_b	1			1
HRV A_pat_c		1		1
HRV A_pat_d		1		1
HRV A_pat_e	2			2
HRV A_pat_f	1			1
HRV A_pat_g	2			2
Subtotal	33	15	16	64

Table 3.28 continued

Species and type	Bintulu	SGH	Sibu	Grand Total
HRV-B (n= 2 types)				
HRV B27	1			1
HRV B69	1		1	2
HRV-C (n= 37 types)				
Cpat_10	2	1	4	7
C35	1	1	3	5
C11	4			4
C12		1	2	3
C24	1		2	3
C41	3			3
C1		1	1	2
C3			2	2
C6	1	1		2
C13		1	1	2
C14	1		1	2
C15	2			2
C25		1	1	2
C38	1		1	2
C43			2	2
C2	1			1
C5			1	1
C7		1		1
C9	1			1
C10		1		1
C17			1	1
C18	1			1
C22		1		1
C27	1			1
C28	1			1
C29			1	1
C30			1	1
C31			1	1
C33			1	1
C36		1		1
C42	1			1
C44	1			1
C45	1			1
C46			1	1
C55		1		1
Cpat_29		1		1
Cpat_19	1			1
Subtotal	25	13	27	65

3.5 Human parainfluenza virus (HPIV)

In this study, of the 902 positive episodes in which at least one virus was identified, HPIV were identified in 104/902 (11.5%) of the total pediatric ALRI recruited episodes and was the fourth most frequently detected virus in our study. Among the total of 104 HPIV-positive episodes, 42.3% were identified from Bintulu hospital, 22.1% from SGH and 35.6% from Sibu hospital (Table 3.29). HPIV-positive episodes detected were differentiated into HPIV types by multiplex PCR assay and further confirmed by sequencing analysis. Among these, the majority were HPIV-3 (n = 60, 57.7%) followed by HPIV-1 (n = 37, 35.6%) and HPIV-4 (n = 7, 6.7%). HPIV-2 was not detected in this study. Patients less than two years old were primarily affected by HPIV-3 but were also affected by HPIV-1 and -4-(Table 3.29). The detection rate of HPIV in children between 2-5 years old was low and therefore an association with certain HPIV type was not possible. The male to female ratio was 1.4:1. Among the HPIV positive episodes, pneumonia was the most common clinical diagnosis (n=82, 78.8%), followed by acute bronchiolitis (n=9, 8.7%). Compared to HPIV-1 and -4, HPIV-3 was detected most frequently from patients with pneumonia (61%, 50/82; $p < 0.05$) while the most predominant type of HPIV detected from patients with viral croup was HPIV-1 (85.7%, 6/7, $p < 0.05$).

Table 3.29: Demographic of patients with Human parainfluenza virus types 1-4

Characteristics	HPIV detection in 1165 hospital recruited episodes			4 types distribution in 104 HPIV-positive episodes			
	HPIV-positive (n= 104)	HPIV-negative (n=1061)	p value*	HPIV-1 (n= 37)	HPIV-3 (n=60)	HPIV-4 (n=7)	p value※
Age group							
1-12 months	64 (61.5)	590 (55.6)	0.245	20 (54.1)	40 (66.7)	4 (57.1)	0.449
13-24 months	27 (26.0)	285 (26.9)	0.843	11 (29.7)	13 (21.7)	3 (42.9)	0.389
25-36 months	6 (5.8)	87 (8.2)	0.383	2 (5.4)	4 (6.7)	0	0.629
3- 5 years	7 (6.7)	99 (9.3)	0.379	4 (10.8)	3 (5.0)	0	0.345
Gender							
Male	60 (57.7)	611(57.6)	0.983	23 (62.2)	33 (55)	4 (57.1)	0.786
Female	44 (42.3)	450 (42.4)	0.983	14 (37.8)	27 (45)	3 (42.9)	0.786
Hospitals							
Bintulu Hospital	44 (42.3)	418 (39.4)	0.563	14 (37.8)	27 (45)	3 (42.9)	0.786
SGH	23 (22.1)	180 (17.0)	0.186	6 (16.2)	15 (25)	2 (28.6)	0.547
Sibu Hospital	37 (35.6)	463(43.6)	0.113	17 (45.9)	18 (30)	2 (28.6)	0.259
Clinical diagnosis							
Pneumonia	82 (78.8)	907 (85.5)	0.071	25 (67.6)	50 (83.3)	7 (100)	0.036
Acute bronchiolitis	9 (8.7)	70 (6.6)	0.426	5 (13.5)	4 (6.7)	0	0.280
Acute exacerbation of bronchial asthma	3 (2.9)	46 (4.3)	0.482	0	3 (5)	0	0.186
Newly diagnosed asthma with concurrent respiratory Tract infection	2 (1.9)	18 (1.7)	0.865	1 (2.7)	1(1.7)	0	0.819
Acute exacerbation of chronic lung disease	0 (0)	10 (0.9)	0.320	0	0	0	-
Viral wheeze	1 (1.0)	8	0.818	0	1(1.7)	0	0.575
Viral croup	7 (6.7)	2	< 0.001	6 (16.2)	1(1.7)	0	0.016

Data are No.(%) of each group.
*Two-tailed Chi-square test/Fisher exact test, testing the distribution of each characteristic between HPIV-positive and HPIV-negative patients.
※Two-tailed Chi-square test/Fisher exact test, testing the distribution of each characteristic between the HPIV types.

The monthly distribution of HPIV types during the period of study is shown in Figure 3.37A. In 2014, few cases of respiratory tract infections were recruited. Most HPIV were detected throughout 2012-2013 season and 2014-2015 season, but HPIV-2 was not detected in any of the episodes. Interestingly, HPIV-1 was mainly detected in 2013-2014 which appeared after that of HPIV-3. HPIV-3 showed an increase in detection during March to September of 2013 and 2015 which coincided with the hot months. On the other hand, only

7 episodes of HPIV-4 were sporadically detected throughout the study period. HPIV-3 was detected in almost two thirds of the HPIV-positive episodes in both Bintulu hospital and SGH (Figure 3.37B & C). However, Sibu hospital had a similar detection rate for both HPIV-1 (46%, 17/37) and HPIV-3 (48.6%, 18/37) (Figure 3.37D).

The results of this study showed a statistically significant association in the HPIV occurrence with meteorological factors. HPIV was positively associated with temperature and inversely correlated with the rainy season ($r= 0.406$, $p = 0.014$; $r= -0.436$, $p = 0.008$) (Figure 3.38).

Of the 104 HPIV-positive episodes, 17 (16.3%) were singly positive for HPIV-1, 21 (20.2%) for HPIV-3 and 3 (2.9%) for HPIV-4. Co-detection of HPIV with other respiratory viruses was observed in 60.6% (63/104) of HPIV-positive episodes. Viruses with the highest frequency of co-detection with HPIV were HAdV (49.2%, 31/63) and RSV (36.5%, 23/63) followed by HRV (17.5%, 11/63). The co-detection rate of HPIV-1 was 54% (20/37), HPIV-3 was 65% (39/60) and HPIV-4 was 57% (4/7). HPIV-3 positive episodes were found to be co-detected with other respiratory viruses, including 29 of dual detection, 9 of triple detection and 1 with quadruple detection involving HCoV, RSV and HRV. HPIV-2 was found to be co-detected with all the other respiratory viruses as well, which involved 18 dual detections and 2 triple detections. It was observed that four HPIV-4 positive episodes were co-detected with other respiratory viruses including 1 with HAdV, 2 with RSV and 1 with HRV (Table 3.30).

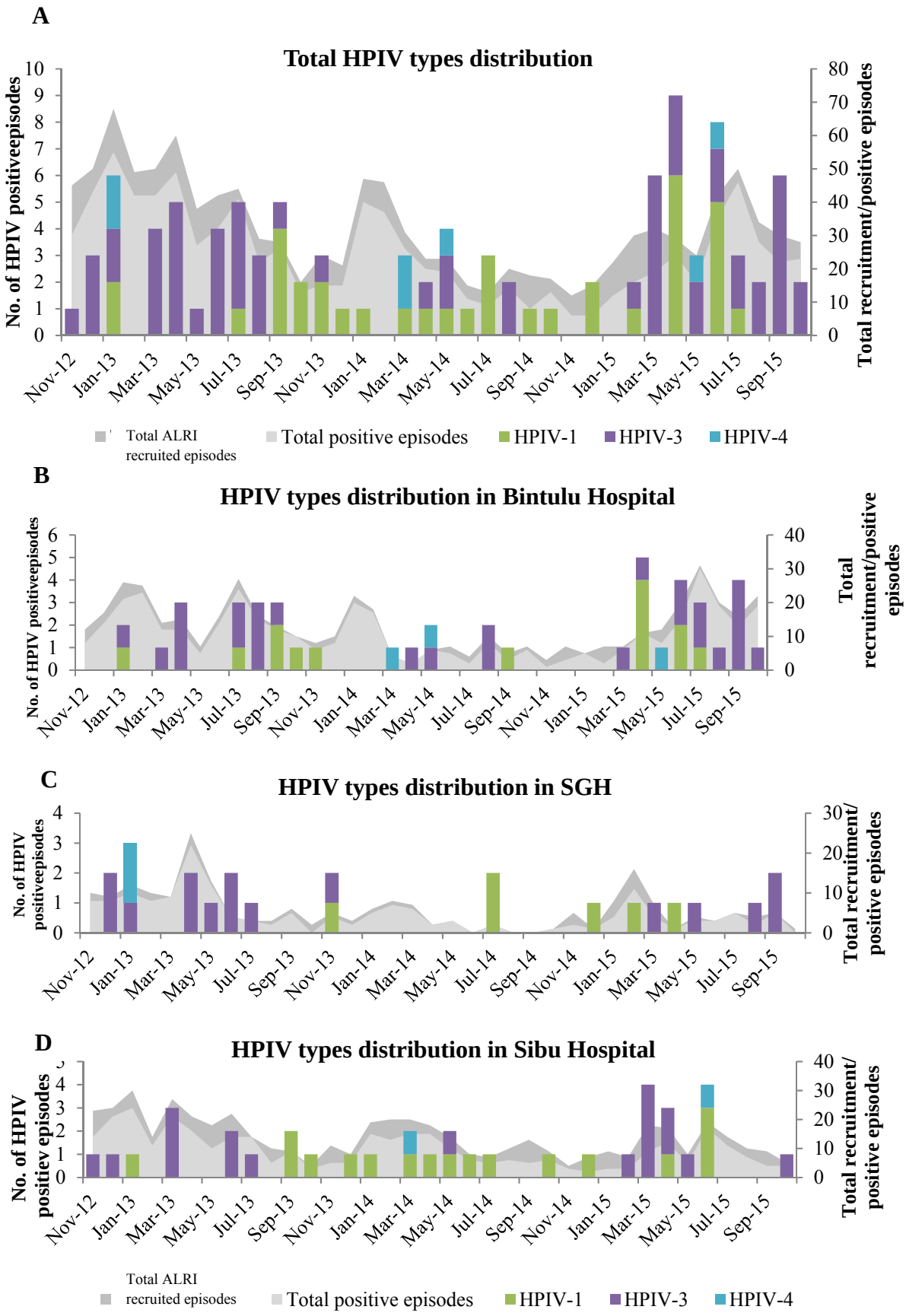


Figure 3.37: Seasonal variations of HPIVs from Nov 2012- Oct 2013 among children < 5 years old with acute respiratory tract infection. * HPIV-2 not found in this study.

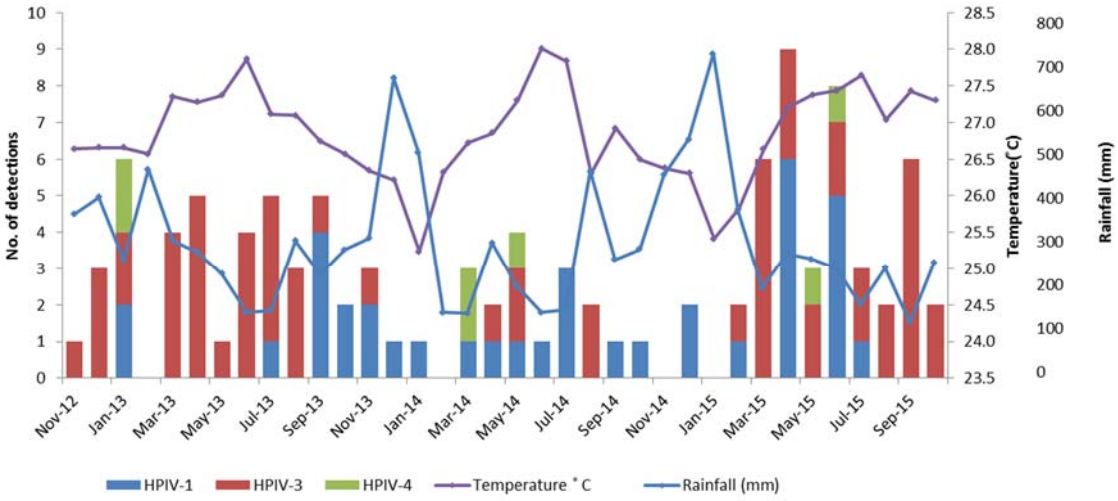


Figure 3.38: HPIV seasonality in association with meteorological factors

Table 3.30: Single and co-detection of HPIV with other respiratory viruses

Type of detection	HPIV-1	HPIV-3	HPIV-4	Total
Single detection	***17***	***21***	***3***	***41***
Co-detection (Total)	***20***	***39***	***4***	***63***
HAdV	2	18	1	21
RSV	8	6	2	16
HRV	2	4	1	7
EV	2	0	0	2
HCoV	1	0	0	1
HMPV	1	0	0	1
HBoV	1	1	0	2
IFV	1	0	0	1
HAdV +RSV	2	3	0	5
HAdV + HRV	0	3	0	3
HAdV + IFV	0	1	0	1
HAdV + HMPV	0	1	0	1
RSV + HCoV	0	1	0	1
RSV + HCoV + HRV	0	1	0	1

3.6 Enteroviruses

Since the discovery of EV-D68 in 1962 among patients with respiratory illness, EV-D68 had been rarely reported until recently, reports of EV-D68 have been documented in association with severe respiratory infections, particularly among children in Asia, Europe and USA (Imamura *et al.*, 2013; Jacobson *et al.*, 2012; Kaida *et al.*, 2011; Lauinger *et al.*, 2012; Tokarz *et al.*, 2012).

During our study period from November 2012 to October 2015, among the 902 positive episodes, a total of 200 were positive by PCR targeting the 5'NTR of HRV/EV. As mentioned in section 3.4, further identification of HRV/EV was confirmed through amplification of the partial VP4/VP2 gene. Of these, 181 were of HRV. Among the remaining 19 positive episodes, 15 episodes showed similarity to EV-D68 by VP4/VP2 sequencing which represented 1.7% of 902 total positive episodes, 1 episode was identified as CVA-A24 and 3 others as CVA-B2, CVA-B4 and CVA-B5 respectively.

Of the 15 EV-D68 positive episodes, 3 (20%) were detected from SGH, 5 (33%) from Bintulu hospital and the remaining 7 from Sibu Hospital. The patients in whom EV-D68 was detected were from 1 month to 2 years in age. Eight were from males and seven from females. EV-D68 was found co-detected with other viruses including HBoV, HAdV and HPIV in four episodes. On the other hand, those detected with coxsackie virus were aged 3 years and below. Three of them were identified from males with one from a female. Three episodes of coxsackievirus were identified from Sibu Hospital while the other one was from Bintulu hospital (Table 3.31). Among these, two were co-detected with HAdV and HPIV respectively.

Figure 3.39 shows the seasonality of EV-D68 and other coxsackieviruses detected throughout this study period. The majority of EV-D68 positive episodes were detected in the

2012-2013 season which accounted for 67% (10/15) of the EV-D68 cases. Two EV-D68 episodes were detected in March and another one in July of 2014. Another two positive episodes of EV-D68 were observed in June and July 2015 respectively. Coxsackieviruses were sporadically detected in March, September and November 2013 and December 2014. Of all the EV-D68 and coxsackievirus detected, most of them were identified in children recruited with a clinical diagnosis of pneumonia (73.7%, 14/19).

VP4/VP2 sequences in GenBank were divided into clades corresponding to VP1 designations (Skowronski *et al.*, 2014). Most (60%, 9/15) of the viruses that were sequenced by VP4/VP2 clustered phylogenetically within clade B with isolates from China (Genebank Accession No. KP240936). The sequences in this branch had high homologies of 97.7-99% with the exception of R824 with homology of 90.7%. Six of the viruses (40%, 6/15) clustered in clade A with homologies between 95-98.5% with isolates from China, Cambodia and USA. In addition, clade A viruses were seen throughout the year, while clade B viruses were more likely to be detected in the 2013-2014 season (Figure 3.40).

After 2014 US outbreak, novel clade and subclades B1 to B3 and D have been identified (Dyrdak et al., 2016; Gong et al., 2016; Yip et al. 2017; Wang et al. 2017). We observed 60% of the viruses clustered within the new subclade B3 with high homologies to isolates from China, Taiwan & Vietnam while 40% clustered in new clade D similar to isolates from Germany & China (Figure 3.41).

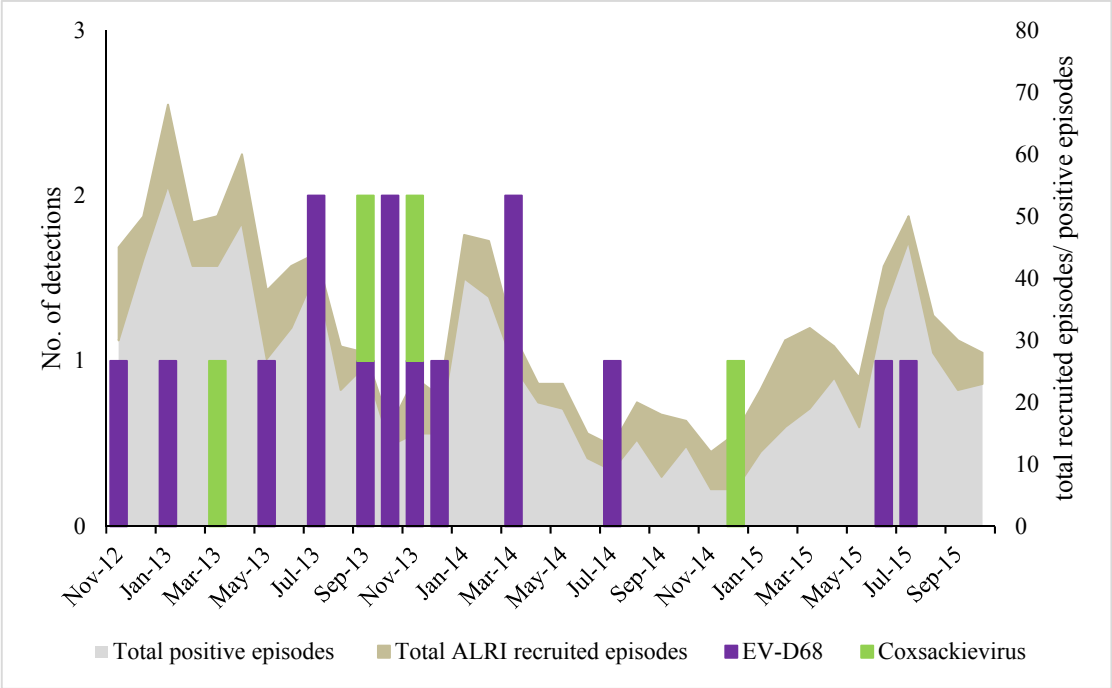


Figure 3.39: Temporal distribution of EV-D68 in Sarawak

Table 3.31: Clinical characteristics and demographic data of the 15 positive episodes of EV-D68 and 4 episodes of Coxsackieviruses in Sarawak, Malaysia.

Sample No.	Hospital	Sex	Age	Date taken	Virus identified	Clinical Diagnosis
R19/1	SGH	M	6 mth 21 d	14-Nov-12	EV-D68, HBoV	Bronchiolitis
R134/1	Bintulu	M	1 yr 6 mths	19-Jan-13	EV-D68	Acute exacerbation of bronchial asthma
R396/1	Sibu	F	2 yr	12-May-13	EV-D68, HAdV B (type 7)	Pneumonia
R517/1	Sibu	M	1 yr 3 mths	14-Jul-13	EV-D68	Pneumonia
R556/1	Sibu	M	2 yr	25-Jul-13	EV-D68, HAdV C (type 2)	Pneumonia
R536/1	Bintulu	M	1 yr 3 mth	5-Oct-13	EV-D68	Pneumonia
R538/1	Bintulu	M	1 mth 29 d	13-Oct-13	EV-D68	Pneumonia
R554/1	Sibu	F	1 yr	27-Nov-13	EV-D68	Pneumonia
R591/1	Bintulu	F	11mths	19-Dec-13	EV-D68	Pneumonia
R499/1	SGH	F	3 mths	Sept-13	EV-D68	Acute bronchiolitis
R765/1	Sibu	M	1 mth	18-Mar-14	EV-D68	Pneumonia
R771/1	Sibu	F	10 mths	30-Mar-14	EV-D68, HPIV-1	Pneumonia
R824/1	Sibu	M	9 mths	1-Jul-14	EV-D68	Pneumonia
R994/1	Bintulu	F	2 mths	3-Jun-15	EV-D68	Pneumonia
R1108/1	SGH	F	1 yr 8 mths	9-Jul-15	EV-D68	Newly diagnosed asthma with concurrent respiratory tract infection
R289/1	Sibu	M	3 yr 10 mths	3-Mar-13	Cox B4	Pneumonia
R531/1	Bintulu	M	1 mth 7 day	20-Sep-13	HAdV B (type 7), Cox B5	Pneumonia
R576/1	Sibu	F	2 mth	17-Nov-13	Cox B2	Pneumonia
R900/1	Sibu	M	6 mth	19-Dec-14	HPIV-1, CoX A24	Acute bronchiolitis

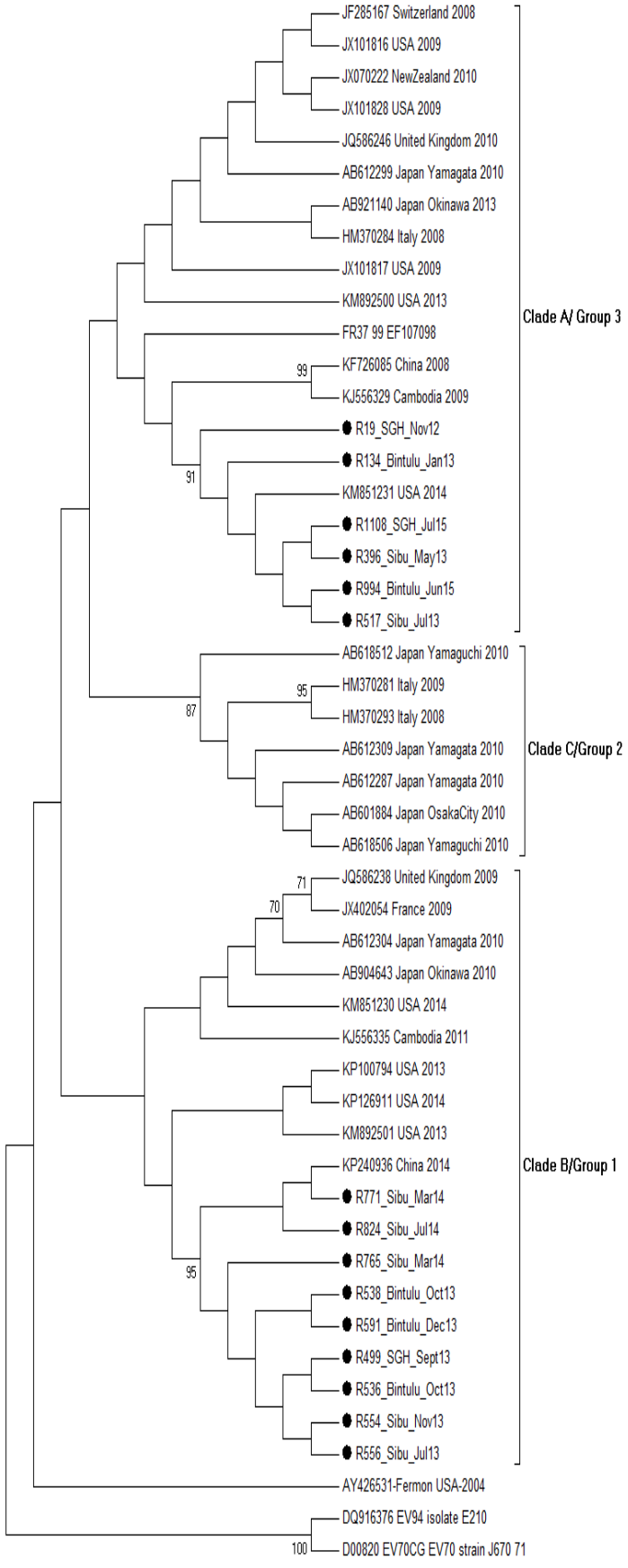


Figure 3.40: Molecular characterization of EV-D68 clinical samples based on phylogenetic analyses of nucleotide sequences of the VP4/VP2 genes. Sequences were aligned using Clustal W version 1.81. (www.clustal.org/). Trees were constructed using the using the maximum-likelihood method with a Jukes-Cantor substitution model as implemented in MEGA 5 software (www.megasoftware.net). Bootstrap values from 1,000 replicates are shown at the nodes. Scale bar indicates number of nucleotide substitutions per site. Global sequences available in GenBank and the Fermon reference strain (GenBank accession no. AY426531) are shown in black. Black dots indicate EV-D68 analyzed in this study. EV70, and EV94 sequences were used as outgroups. Phylogenetic clusters are labelled clade A, B, and C as described in Tokarz *et al.*, (2012), corresponding to major group 3, 1, and 2 as described in Meijer *et al.*, (2012) and lineage 2, sub-lineage 1.2, and sub-lineage 1.1 as described in Lauinger *et al.*, (2012) respectively.

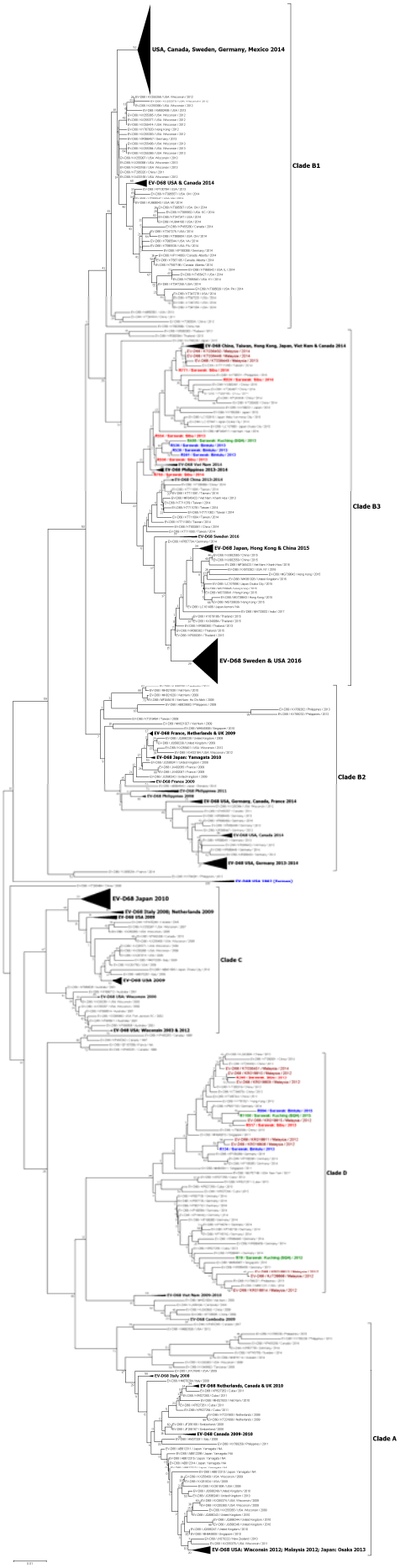


Figure 3.41: (A) Phylogenetic tree of Enterovirus D68 based on nucleotide sequences of the VP4/VP2 genes which present 4 major clades A to D. Clade B was further divided into B1 to B3. Neighbour-joining trees were constructed and the distance was calculated according to Kimura's two-parameter method, with labelling of branches showing at least 70% bootstrap support. The coloured code indicates the sampling site: blue, Bintulu Hospital; red, Sibu Hospital; green, SGH. The node label includes the name, country and year of strains separated by vertical bars.

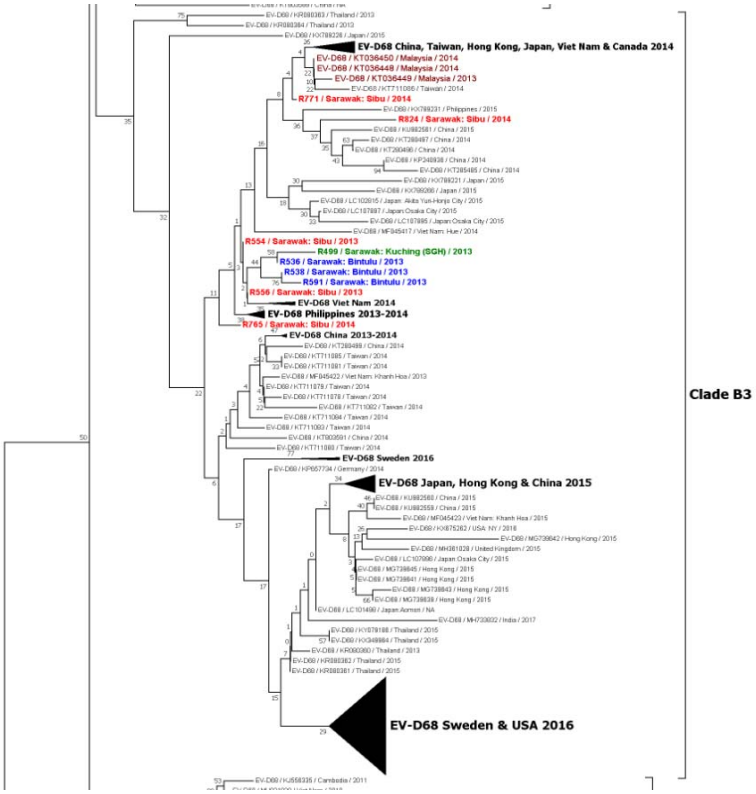


Figure 3.41: (B) Enlarged phylogenetic tree of EV-D68 subclade B3. The coloured code indicates the sampling site: blue, Bintulu Hospital; red, Sibu Hospital; green, SGH. The node label includes the name, country and year of strains separated by vertical bars.

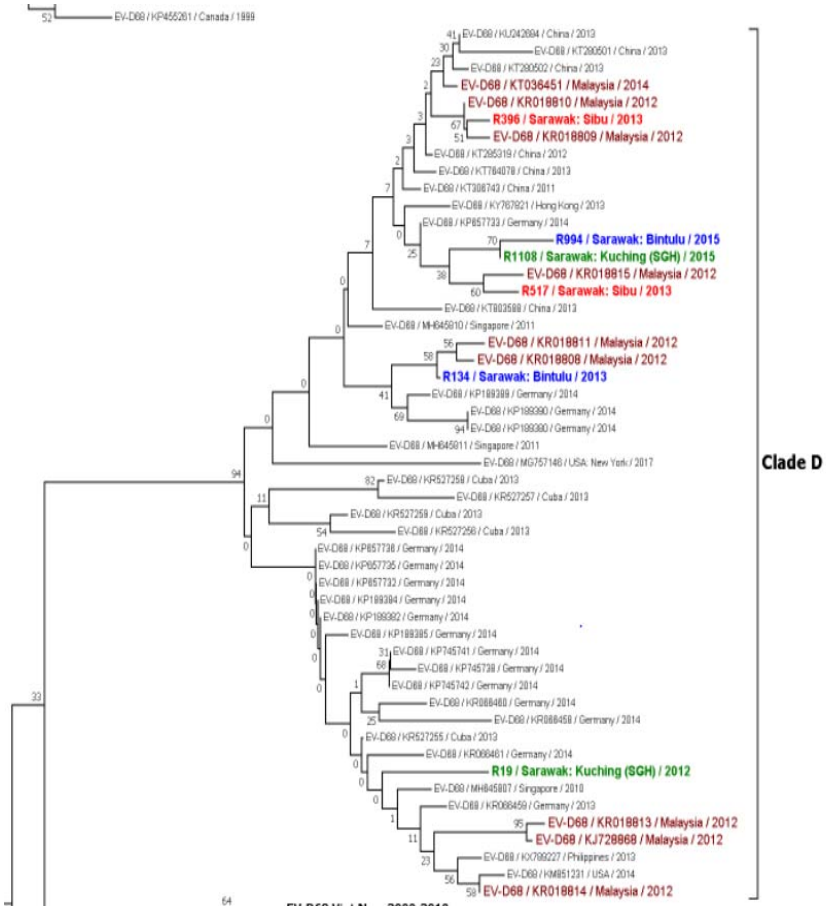


Figure 3.41: (C) Enlarged phylogenetic tree of EV-D68 clade D. The coloured code indicates the sampling site: blue, Bintulu Hospital; red, Sibu Hospital; green, SGH. The node label includes the name, country and year of strains separated by vertical bars.

3.7 Human metapneumovirus (HMPV)

As mentioned briefly in section 3.1, HMPV was detected in 51 (5.7%) of the 902 total positive episodes among children below 5 years old admitted to PICU. HMPV was not significantly found among different age group with a detection rate 4.5% to 6.7% (Table 3.32). The male to female ratio was 1.4. Similar to other respiratory viruses detected in this study, pneumonia was the most common clinical diagnosis (n=42, 5.6%), followed by acute exacerbation of bronchial asthma (n=4, 9.5%) and acute bronchiolitis (n=3, 4.5%).

Table 3.32: Demographic of patients with HMPV

Characteristics	**Total Positive episodes (n= 902)**	**HMPV Positive (n=51)**
Age group		
1-12 months	535(59.3)	28 (5.23)
13-24 months	240(26.6)	16 (6.7)
25-36 months	66 (7.3)	3 (4.5)
3- 5 years	61 (6.8)	4 (6.6)
Gender		
Female	366 (40.6)	21 (5.7)
Male	536 (59.4)	30 (5.6)
Hospitals		
Bintulu Hospital	389 (43.1)	31(8.0)
SGH	164 (18.1)	7 (4.3)
Sibu Hospital	349 (38.7)	13 (3.7)
Clinical Diagnosis		
Pneumonia	751 (64.5)	42 (5.6)
Acute bronchiolitis	67 (5.8)	3 (4.5)
Acute exacerbation of bronchial asthma	42 (3.6)	4 (9.5)
Newly diagnosed asthma with concurrent respiratory tract infection	19 (1.6)	0 (0)
Acute exacerbation of chronic lung disease	6 (0.5)	0 (0)
Viral wheeze	8 (0.7)	1 (12.5)
Viral croup	9 (0.8)	1 (11.1)

Prevalence by year was reported as 6.9% in 2012-2013 season, 5.7% in 2013-2014 season and decreased to 3.6% in 2014-2015 season (Table 3.33). More than half of HMPV positive episodes were identified in the 2012-2013 season (29/51, 56.9%). As shown in Figure 3.42, there was an increase of HMPV detection from April to September 2013. To

study the seasonal distribution of HMPV in the three study hospitals, the total HMPV detections from respiratory samples collected during the months of October 2012 to November 2015 were included in Figure 3.43. HMPV highest detection rate was observed in April 2013. Bintulu hospital contributed to the highest HMPV detection with 17 positive episodes identified in 2012-2013 season, followed by 8 positive episodes in Sibu hospital and 4 positive episodes in SGH during the 2012-2013 season. For the next two seasons of study, HMPV were mainly detected from Bintulu hospital with few other positive episodes detected sporadically from Sibu Hospital and SGH.

Table 3.33: Total HMPV distribution by month/year

	1st season (2012-2013)	2nd season (2013-2014)	3rd season (2014-2015)	Whole study period	percentage
Total positive episodes	422	227	253	902	
HMPV Total	29 (6.9%)	13 (5.7%)	9 (3.6%)	51(5.6%)	100
Nov	0	0	1	1	1.96%
Dec	1	2	0	3	4.69%
Jan	0	1	1	2	1.87%
Feb	0	2	1	3	3.16%
Mar	1	1	0	2	2.30%
Apr	9	0	0	9	9.68%
May	3	2	0	5	8.06%
Jun	4	2	1	7	8.97%
Jul	4	0	1	5	5.21%
Aug	4	1	1	6	9.38%
Sep	3	0	1	4	7.14%
Oct	0	2	2	4	8.16%

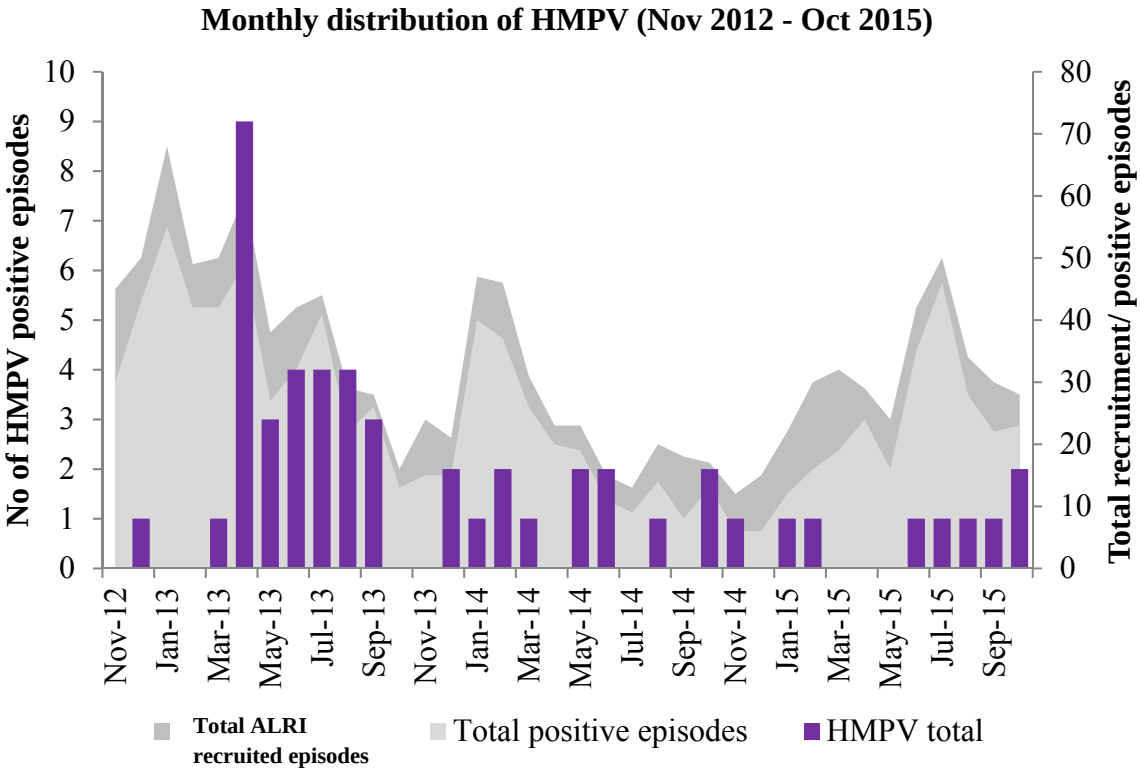


Figure 3.42: Total monthly HMPV distribution (Nov 2012- Oct 2015)

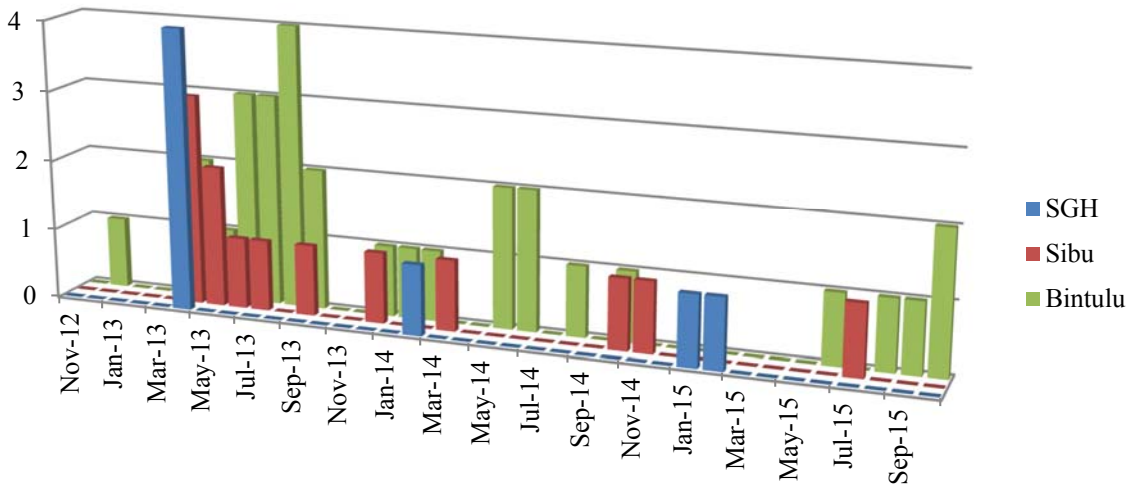


Figure 3.43: Monthly HMPV distribution among three hospitals (Nov 2012 – Oct 2015)

HMPV was singly detected in 28 (54.9%) of the total HMPV positive episodes while 23 (45.1%) were co-detected with HAdV, RSV, HPIV and HRV. Among the co-detected episodes, only one RSV was co-detected with HMPV, two involving co-detection with HPIV and 21 (91.3%) were mainly co-detected with HAdV (Table 3.34).

Table 3.34: Single and co-detection of HMPV with other respiratory viruses

Type of detection	HMPV
Single detection	***28***
Co-detection (Total)	***23***
HAdV	19
RSV	1
HPIV	1
HAdV + HPIV	1
HAdV + HRV	1

3.8 Human coronavirus (HCoV)

Overall, 27 episodes of coronavirus (3.0%) were detected. Of the HCoV positive episodes, the majority were HCoV-OC43 (n=10, 0.86%), followed by HCoV-229E (n=9, 0.77%) and HCoV-NL63 (n=8, 0.69%). Most (59%) of the HCoV detections were from patients aged 1-12 months while 30% were from those aged 13-24 months and 11% were of 25-36 months old. HCoV were equally distributed between male and females. Among the 27 HCoV positive episodes, 41% were identified from Bintulu hospital, 26% from SGH and 33% from Sibu hospital. As shown in Table 3.35, pneumonia was the most common clinical diagnosis (n=23, 85%), followed by acute bronchiolitis (n=2, 7.4%), acute exacerbation of bronchial asthma (n=1, 3.7%) and viral croup (n=1, 3.7%). The clinical characteristics and demographic data of each 27 HCoV-positive episodes are listed in Table 3.36.

Table 3.35: Demographic of patients with Human coronavirus

Characteristics	HCoV detection in 1165 ALRI recruited episodes			HcoV types distribution in 27 positive episodes			
	HCoV positive (n= 27)	HCoV negative (n=1138)	p value*	HCoV-NL63 (n=8)	HCoV-OC43 (n=10)	HCoV-229E (n=9)	p value※
Age group							
1-12 months	16 (59)	638 (56)	0.741	5 (62.5)	6 (60)	5 (55.6)	0.957
13-24 months	8 (30)	304 (27)	0.735	2 (25)	3 (30)	3 (33.3)	0.931
25-36 months	3 (11)	90 (8)	0.564	1(12.5)	1 (10)	1 (11.1)	0.986
3- 5 years	0 (0)	106 (9)	0.096	0	0	0	-
Gender							
Male	14 (52)	657 (58)	0.541	5 (62.5)	7 (70)	2 (22.2)	0.08
Female	13 (48)	481 (42)	0.541	3 (37.5)	3 (30)	7 (77.8)	0.08
Hospitals							
Bintulu Hospital	11 (41)	451(40)	0.907	3 (37.5)	6 (60)	2 (22.2)	0.232
SGH	7 (26)	196 (17)	0.264	3 (37.5)	1 (10)	3 (33.3)	0.307
Sibu Hospital	9 (33)	491 (43)	0.309	2 (25)	3 (30)	4 (44.4)	0.673
Clinical diagnosis							
Pneumonia	23 (85)	966 (85)	0.966	6 (75)	10 (100)	7 (77.8)	0.127
Acute bronchiolitis	2 (7.4)	77 (7)	0.897	2 (25)	0	0	0.072
Acute exacerbation of bronchial asthma	1 (3.7)	48 (4)	0.893	0	0	1 (11.1)	0.321
Newly diagnosed asthma with concurrent respiratory tract infection	0 (0)	20 (2)	0.331	0	0	0	-
Acute exacerbation of chronic lung disease	0 (0)	10 (0.9)	0.493	0	0	0	-
Viral wheeze	0 (0)	9 (0.8)	0.515	0	0	0	-
Viral croup	1 (3.7)	8 (0.7)	0.118	0	0	1 (11.1)	0.321

Data are No.(%) of each group.
*Two-tailed Chi-square test/Fisher exact test, testing the distribution of each characteristic between HCoV-positive and HCoV-negative patients.
※Two-tailed Chi-square test/Fisher exact test, testing the distribution of each characteristic between the HCoV types

Table 3.36: Clinical characteristics and demographic data of the 27 positive episodes of HCoV in Sarawak, Malaysia.

Sample No.	Hospital	Sex	Age	Date taken	Clinical Diagnosis	Primary sequencing (BLAST)
R34/1	SGH	M	1 yr 2 mths	28-Nov-12	Acute Bronchiolitis	HCoV-NL63
R74/1	Bintulu	F	2 mths 20 days	22-Dec-12	Pneumonia	HCoV-NL63; AdV D (type 8); RSV B
R114/1	Sibu	M	10 mths	14-Dec-12	Pneumonia	HRV C, HCoV-NL63
R250/1	SGH	M	10 mths	8-Apr-13	Pneumonia	HCoV_229E
R299/1	Sibu	F	2 mths	12-Mar-13	Pneumonia	RSV B, HCoV-229E
R304/1	Sibu	F	2 yr	19-Mar-13	Pneumonia	HCoV-OC43
R324/1	Bintulu	F	1 mth 4 d	5-May-13	Pneumonia	HCoV_229E
R396/1	Sibu	F	1 yr 9 mths	12-May-13	Pneumonia	AdV B (type 7), EV 68, HCoV-229E
R426/3	Bintulu	M	7 mth	22-Jul-13	Pneumonia	HCoV-OC43
R440/1	Sibu	F	2 yr 8 mths	11-Jun-13	Pneumonia	HCoV-229E
R521/1	Sibu	M	8 mths	19-Jul-13	Pneumonia	AdV B (type 7); HCoV-OC43, HRV B
R535/1	Bintulu	M	2 yr 2 mth	3-Oct-13	Pneumonia	HCoV-OC43
R663/1	Sibu	F	4mth	4-Feb-14	Pneumonia	HCoV-OC43
R750/1	SGH	F	10 mth	30-Jul-14	Viral Croup	HCoV_229E, HPIV-1
R826/1	Sibu	F	2 mth	4-Aug-14	Pneumonia	RSV A, HCoV-229E
R847/1	Bintulu	M	1 yr 13 day	1-Dec-14	Pneumonia	RSV A, HCoV-OC43
R858/1	SGH	M	4 mth 23 days	14-Jan-15	Pneumonia , TRO Pertussis	HCoV-OC43
R920/1	Bintulu	M	1 mth 2 weeks	21-Jan-15	Pneumonia	HCoV-OC43
R1003/1	Bintulu	M	4 mth	22-Jun-15	Pneumonia	HCoV-NL63
R1008/1	Bintulu	M	3 mth 25 days	28-Jun-15	Pneumonia	HCoV-OC43, RSV B
R1100/1	Bintulu	F	1 yr 1 mth	30-Jul-15	Acute exacerbation of bronchial asthma sec to RTI	RSV A, HCoV-229E
R1112/1	SGH	M	1 yr 17 day	26-Jul-15	Pneumonia	HCoV-229E, HBoV, RSV B, HAdV B (Type 3)
R1123/1	Bintulu	M	6 weeks	18-Aug-15	Pneumonia	HRV C, HAdV B (type 3), HCoV-NL63
R1142/1	SGH	M	1 yr 1 mth	31-Aug-15	Acute Bronchiolitis	RSV A, HCoV-NL63, H PIV-3, HRV C
R1143/1	SGH	F	6 mth	4-Sep-15	Pneumonia	RSV A, HCoV-NL63, HPIV-3
R1152/1	Bintulu	F	1 yr 11 mths	21-Sep-15	Pneumonia	HCoV-OC43
R1183/1	Sibu	F	2 yr 3 mth	20-Jul-15	Pneumonia	HAdV B (type 7), RSV A, HCoV-NL63

The seasonal distribution of HCoV is shown in Figure 3.44. There were no seasonal peaks associated with HCoV observed during the three-year study period. HCoV-OC43 was sporadically identified in ten positive episodes throughout the study period with most of the cases detected from Bintulu Hospital (n= 6) followed by Sibu hospital (n=3) and SGH (n=1). HCoV-229E activity was also sporadic throughout, with no obvious seasonal distribution. Among the nine HCoV-229E positive episodes, Sibu Hospital presented a higher frequency of HCoV-229E detection (n= 4) followed by SGH (n=3) and Bintulu hospital (n=2). HCoV-NL63 was found circulating in November-December 2012, was not detected further but reappeared in June-September 2015.

High rates of HCoV co-detection with other respiratory viruses were observed (Table 3.37). Of the 27 HCoV positive episodes, 15 (55.6%) were co-detected with at least one other respiratory viruses, six with two other viruses and two with three other viruses. Most HCoV co-detection involved RSV which accounted for 66.7% of the HCoV co-detected viruses.

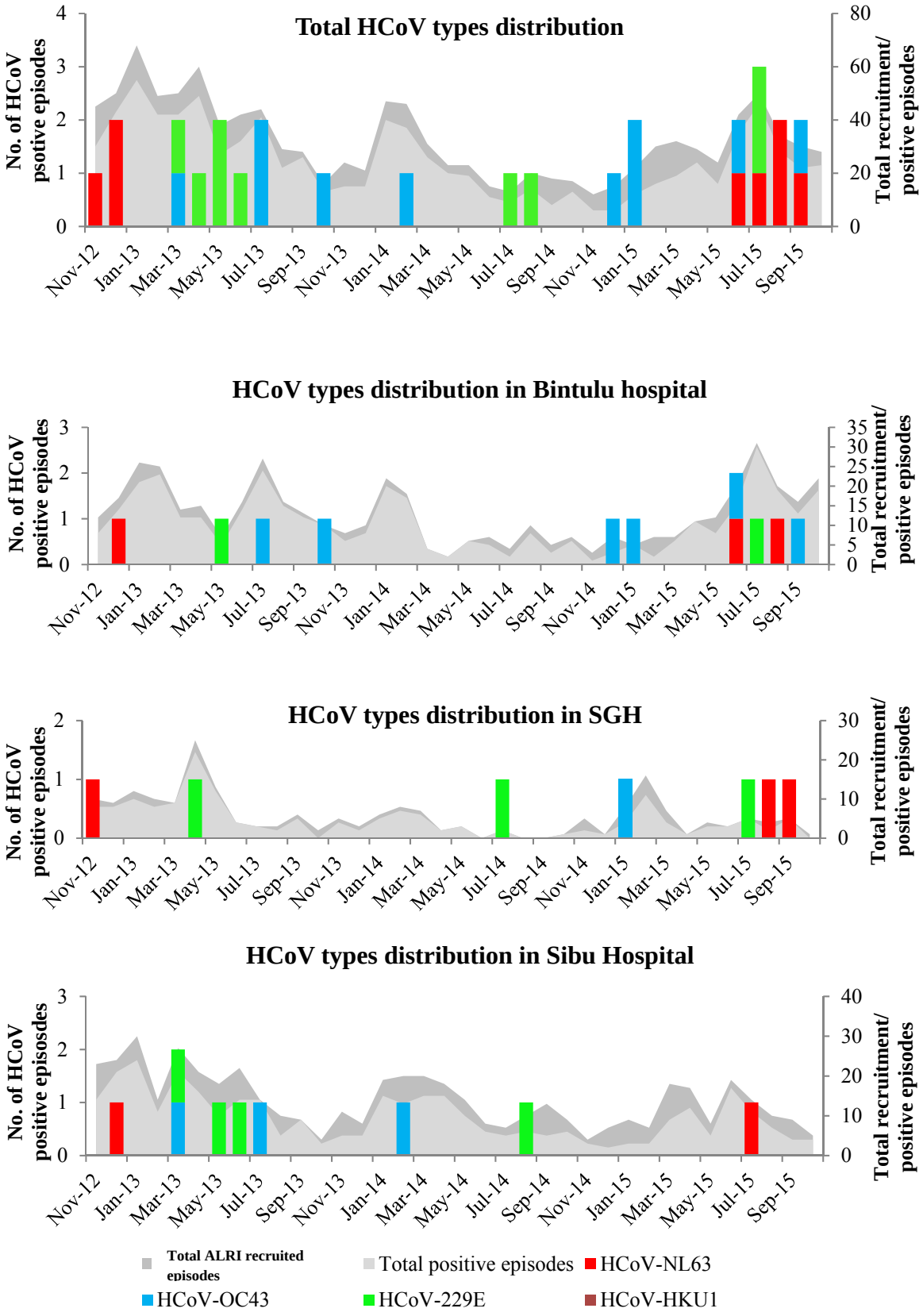


Figure 3.44: Seasonal variations of HCoV from Nov 2012- Oct 2013 among children < 5 years old with acute respiratory tract infection. * HCoV-HKU1 not found in this study.

Table 3.37: Single and co-detection of HCoV with other respiratory viruses

Type of detection	HCoV-NL63	HCoV-OC43	HCoV-229E	Total
Single detection	***2***	***7***	***3***	***12***
Co-detection (Total)	***6***	***3***	***6***	***15***
HRV	1	0	0	1
RSV	0	2	3	5
HPIV	0	0	1	1
HAdV +RSV	2	0	0	2
HAdV + HRV/EV	1	1	1	3
RSV+HPIV	1	0	0	1
RSV + HPIV + HRV	1	0	0	1
HAdV+RSV +HBoV	0	0	1	1

3.9 Human bocavirus (HBoV)

HBoV was detected in 26 (2.9%) of 902 total positive episodes. A significant detection rate was observed among those aged 1-12 months and 13-24 months which accounted for 92% of the total HBoV positive episodes. Of the 26 children with positive HBoV, the male to female ratio was 1.4. The HBoV distribution was similar among three study hospitals which ranged between 27%-39%. The clinical diagnoses associated with the 26 episodes of HBoV detection are shown in Table 3.38. The most common clinical diagnosis was pneumonia (73.1%, 19/26), followed by acute bronchiolitis (11.5%, 3/26), acute exacerbation of bronchial asthma (7.7%, 2/26), newly diagnosed asthma with concurrent respiratory tract infection (3.8%, 1/26) and acute exacerbation of chronic lung disease (3.8%, 1/26). The clinical characteristics and demographic data of each 26 HBoV positive episodes are listed in Table 3.39. Figure 3.45 shows the temporal distribution of HBoV during the study period. The seasonal distribution of HBoV showed that they were detected sporadically during the study period in the three study hospitals (Figure 3.46).

HBoV alone was detected in 34.6% (9/26) of the total HBoV positive episodes. The remaining 65.4% (17/26) was detected concurrently with other respiratory viruses. Of those co-detected viruses, it was observed that 65% (11/17) involved HAdV or RSV (Table 3.40). This high rate of co-detection was mainly seen in children less than 2 years old.

Table 3.38: Demographic of patients with HBoV

Characteristics	**Total positive episodes (n= 902)**	**HBoV Positive (n=26)**	**P value**
Age group			
1-12 months	535(59.3)	9 (1.7)	***0.009***
13-24 months	240(26.6)	15 (6.3)	***0.000***
25-36 months	66 (7.3)	2 (3.0)	0.846
3- 5 years	61 (6.8)	0	0.132
Gender			
Female	366 (40.6)	11 (3.0)	0.995
Male	536 (59.4)	15 (2.8)	0.995
Hospitals			
Bintulu Hospital	389 (43.1)	10 (2.6)	0.90
SGH	164 (18.1)	7 (4.3)	0.231
Sibu Hospital	349 (38.7)	9 (2.6)	0.357
Clinical Diagnosis			
Pneumonia	751 (64.5)	19 (2.5)	0.123
Acute bronchiolitis	67 (5.8)	3 (4.5)	0.384
Acute exacerbation of bronchial asthma	42 (3.6)	2 (4.8)	0.532
Newly diagnosed asthma with concurrent respiratory tract infection	19 (1.6)	1 (5.3)	0.476
Acute exacerbation of chronic lung disease	6 (0.5)	1 (16.7)	0.267
Viral wheeze	8 (0.7)	0	0.556
Viral croup	9 (0.8)	0	0.556

P-value was calculated using Fisher Exact test

Table 3.39: Clinical characteristics and demographic data of the 26 positive episodes of HBoV in Sarawak, Malaysia.

Sample No.	Hospital	Sex	Age	Date taken	Clinical Diagnosis	Primary sequencing (BLAST)
R19/1	SGH	M	6 mths 21 d	14-Nov-12	Bronchiolitis	HBoV, EV 68, ADV C (type 2)
R28/1	SGH	M	1 yr 7 mths	21-Nov-12	Acute exacerbation of bronchial asthma	HRV A (cell culture), HBoV unsequenced
R40/1	Sibu	F	11 mths	16-Nov-12	Acute bronchiolitis	PIV 3, HBOV
R42/1	Sibu	F	1 yr 6 mths	26-Nov-12	Pneumonia	HBoV
R199/1	Bintulu	F	1 yr 1o mths	9-Feb-13	Pneumonia	RSV B, HBoV
R265/1	SGH	F	5 mths	15-Apr-13	Pneumonia	AdV C (type 5), HBoV
R491/1-R	SGH	M	8 mths	10-Sep-13	Pneumonia	AdV B (type 7), HBoV
R500/1	SGH	M	1 yr 3 mths	1-Sep-13	Acute exacerbation of bronchial asthma secondary to RTI	HBoV
R569/1	Sibu	M	1 yr 4 mths	1-Sep-13	Pneumonia	HBoV
R573/1	Sibu	F	13 mths	22-Sep-13	Pneumonia	AdV B (type 7), HBoV
R575/1	Sibu	M	1 yr 1 mths	25-Sep-13	Pneumonia	HBoV
R578/1	Sibu	F	2 yr 6 mths	18-Nov-13	Pneumonia	HRV B, HBoV
R581/1	Bintulu	F	1 yr 11 mths	28-Nov-13	Pneumonia	RSV A, HBoV
R599/1	Bintulu	M	6 mths	28-Dec-13	Pneumonia	HBoV
R652/1	Bintulu	M	7mths	20-Feb-14	Pneumonia	RSV A, HBoV
R656/1	Bintulu	F	1 yr 7mths	1-Mar-14	Pneumonia	RSV A, HBoV
R697/1	SGH	M	1 yr 1 mths	16-Apr-14	Acute bronchiolitis	HBoV
R789/1	Bintulu	M	1 yr 3 mths	12-Sep-14	Pneumonia	PIV 1, HBoV
R829/1	Sibu	F	9 mths	16-Aug-14	Pneumonia	HBoV
R833/1	Sibu	M	8 mths	25-Aug-14	Pneumonia	HBoV
R867/1	Bintulu	F	2 yr 2 mths	3-Jan-15	Pneumonia	IBV, HBoV
R877/1	Sibu	M	1yr 5 mths	19-Sep-14	Pneumonia	HBoV
R986/1	Bintulu	F	1 yr 2 mths	13-May-15	Newly diagnosed bronchial asthma with concurrent respiratory tract infection	HBoV, AdV B (type 3)
R1020/1	Bintulu	M	8 mths	5-Jul-15	Pneumonia	HRV A, ADV C (Type 1), HBoV
R1086/1	Bintulu	M	1 yr 10 mths	16-Jul-15	Exacerbation of chronic lung Disease	ADV B(Type 3), HBoV

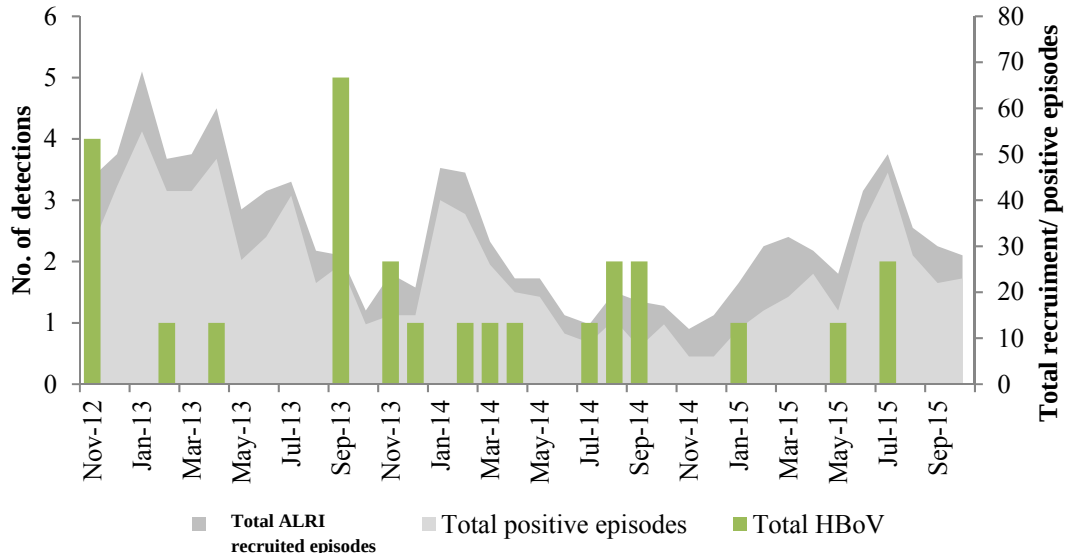


Figure 3.45: Temporal distribution of HBoV in Sarawak, November 2012-October 2015

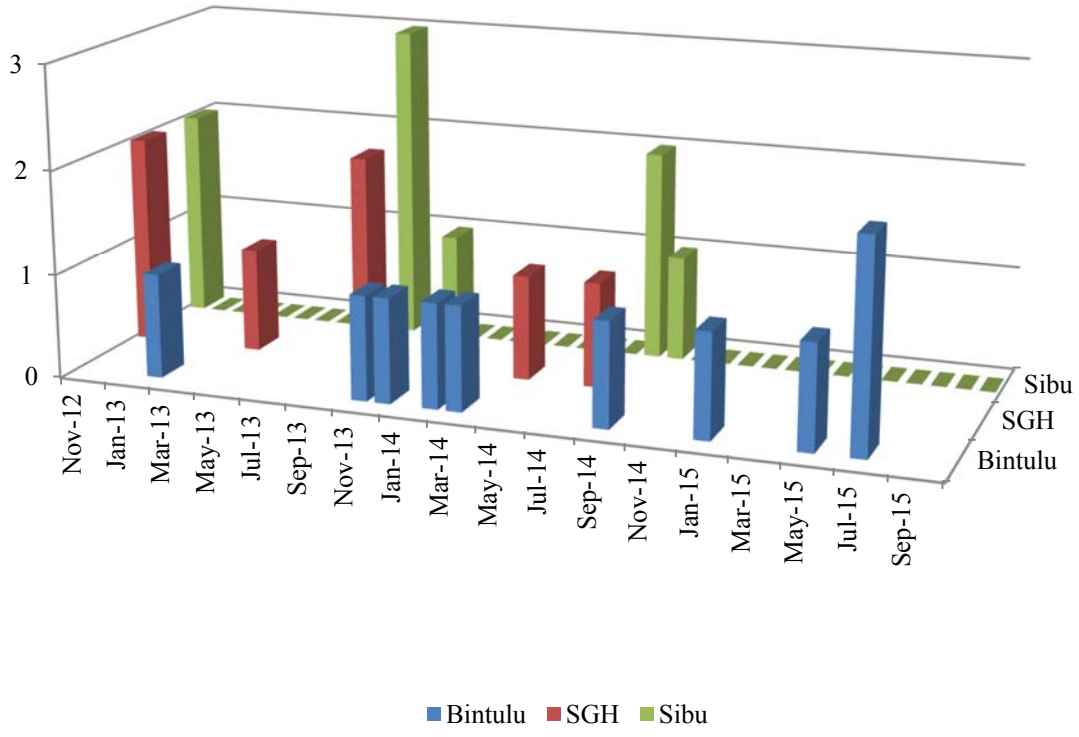


Figure 3.46: Monthly HBoV distribution among three hospitals (Nov 2012 – Oct 2015)

Table 3.40: Single and co-detection of HBoV with other respiratory viruses

Type of detection	HBoV
Single detection	***9***
Co-detection (Total)	***17***
HAdV	5
RSV	4
HRV/EV	3
HPIV	2
IFV	1
HAdV + HRV	1
HAdV + RSV + HCoV	1

3.10 Influenza virus (IFV)

IFV was detected in 22 (2.4%) of 902 total positive episodes. No significant detection rate was observed among the different age groups. The male to female ratio of IFV was 1.4. The IFV distribution was similar between Bintulu and Sibu hospital with a detection rate ranged between 2.1% to 2.9%. IFV was significantly found among those with acute bronchiolitis which accounted for 7.5% (Table 3.41). The clinical characteristics and demographic data of each 22 IFV positive episodes are listed in Table 3.42. IBV was detected in 50% of the 22 positive episodes followed by IAV in 45.5%. Only one positive episodes of ICV was detected throughout the three-year study period. The seasonal distribution of IFV was similar to that of HBoV which showed that they were detected sporadically during the study period in the three study hospitals (Figure 3.47). Interestingly, SGH only detected four episodes of IBV while Sibu hospital detected 4 episodes of IAV and 3 episodes of IBV. Three types of IFV was identified for samples from Bintulu hospital which included 3 of IAV, 4 of IBV and 1 of ICV.

Table 3.41: Demographic of patients with IFV

Characteristics	IFV detection in 902 ALRI positive episodes			IFV types distribution in 22 IFV-positive episodes (% by IFV-positive)			
	IFV-positive (n= 22)	IFV-negative (n=880)	p value*	IAV (n= 10)	IBV (n=11)	ICV (n=1)	p value※
Age group							
1-12 months (n= 535)	12 (2.2)	523 (97.8)	0.645	5 (41.7)	6 (50.0)	1 (8.3)	0.523
13-24 months (n= 240)	6 (2.5)	234 (97.5)	0.943	5 (83.3)	1 (16.7)	0	0.074
25-36 months (n= 66)	2 (3.0)	64 (97.0)	0.672	0	2 (100)	0	0.226
3- 5 years (n=61)	2 (3.3)	59 (96.7)	0.656	0	2 (100)	0	0.226
Gender							
Male (n= 366)	10 (2.7)	356 (97.3)	0.637	5 (50.0)	4 (40.0)	1 (10.0)	0.362
Female (n=536)	12 (2.2)	524 (97.8)	0.637	5 (50.0)	7 (58.3)	0	0.362
Hospitals							
Bintulu Hospital (n=389)	8 (2.1)	381 (97.9)	0.517	3 (37.5)	4 (50.0)	1 (12.5)	0.332
SGH (n=164)	4 (2.4)	160 (97.6)	0.925	0	4 (100)	0	0.040
Sibu Hospital (n= 349)	10 (2.9)	339 (97.1)	0.808	7 (70.0)	3 (30.0)	0	0.074
Clinical diagnosis							
Pneumonia (n=751)	15 (2.0)	736 (98.0)	0.055	9 (60.0)	6 (40.0)	0	0.053
Acute bronchiolitis (n=67)	5 (7.5)	62 (92.5)	**0.019**	1 (20.0)	3 (60.0)	1 (20.0)	0.123
Acute exacerbation of bronchial asthma (n=42)	2 (4.8)	40 (95.2)	0.318	0	2 (100.0)	0	0.226
Newly Diagnosed Asthma with Concurrent Respiratory Tract Infection (n=19)	0	19 (100.0)	0.486	0	0	0	-
Acute Exacerbation of chronic lung disease (n=6)	0	6 (100.0)	0.698	0	0	0	-
Viral Wheeze (n=8)	0	8 (100.0)	0.653	0	0	0	-
Viral Croup (n=9)	0	9 (100.0)	0.634	0	0	0	-

Data are No.(%) of each group.

*Two-tailed Chi-square test/Fisher exact test, testing the distribution of each characteristic between IFV-positive and IFV-negative patients.

※Two-tailed Chi-square test/Fisher exact test, testing the distribution of each characteristic between the IFV types

Table 3.42: Clinical characteristics and demographic data of the 22 positive episodes of IFV in Sarawak, Malaysia

Sample No.	Hospital	Sex	Age	Date taken	Clinical Diagnosis	Primary sequencing Results (BLAST)
R44/1	Sibu	F	6 mths	27-Nov-12	Pneumonia	IAV, HAdV B (type 7)
R52/1	Sibu	M	1 yr 1 mth	8-Nov-12	Pneumonia	IAV
R140/1	SGH	F	1 yr	26-Jan-13	Acute exacerbation of bronchial asthma	HRV A, IBV
R149/1	Sibu	M	2 yrs 3 mths	2-Jan-13	Pneumonia	IBV
R159/1	Sibu	M	7 mths	10-Jan-13	Pneumonia	IAV
R207/1	Bintulu	M	7 mths	17-Feb-13	Acute bronchiolitis	HAdV B (type 7); ICV; RSV B
R266/1	SGH	F	4 yr 7mth	20-Apr-13	Pneumonia	IBV
R303/1	Sibu	F	5 mth	17-Mar-13	Acute bronchiolitis	HAdV B (Type 7), IBV
R310/1	Bintulu	F	4 yr	16-Apr-13	Pneumonia	IBV
R448/1	Sibu	F	5 mth	13-Jun-13	Pneumonia	HAdV D (type 36). IBV
R514/1	Sibu	M	1 yr 1 mth	9-Jul-13	Pneumonia	HAdV B (type 7); HPIV 3; IAV
R542/1	Bintulu	F	1 yr 7 mths	19-Oct-13	Pneumonia	IAV
R562/1	Sibu	M	1 mth	13-Nov-13	Pneumonia	IAV
R589/1	Bintulu	M	6 mth	14-Dec-13	Acute bronchiolitis	IBV
R593/1	Bintulu	M	7 weeks	21-Dec-13	Acute bronchiolitis	HAdV B (type 7), IAV
R621/1	Bintulu	F	1 yr 9 mth	25-Jan-14	Pneumonia	IAV
R632/1	SGH	M	1 yr 3 mths	10-Feb-14	Acute exacerbation of bronchial asthma	IBV
R646/1	Bintulu	M	10 mth	18-Feb-14	Pneumonia	IBV
R729/1	Sibu	F	5mth	12-Feb-14	Pneumonia	***IAV**
R776/1	Sibu	F	2 yrs	8-Apr-14	Pneumonia	HRV C, IAV
R867/1	Bintulu	F	2 yr 2 mths	3-Jan-15	Pneumonia	IBV, HBoV
R923/1	SGH	F	3 mths	1-Feb-15	Acute bronchiolitis	IBV, HPIV 1

* IAV was detected positive by gel electrophoresis but not confirmed by sequencing blast search

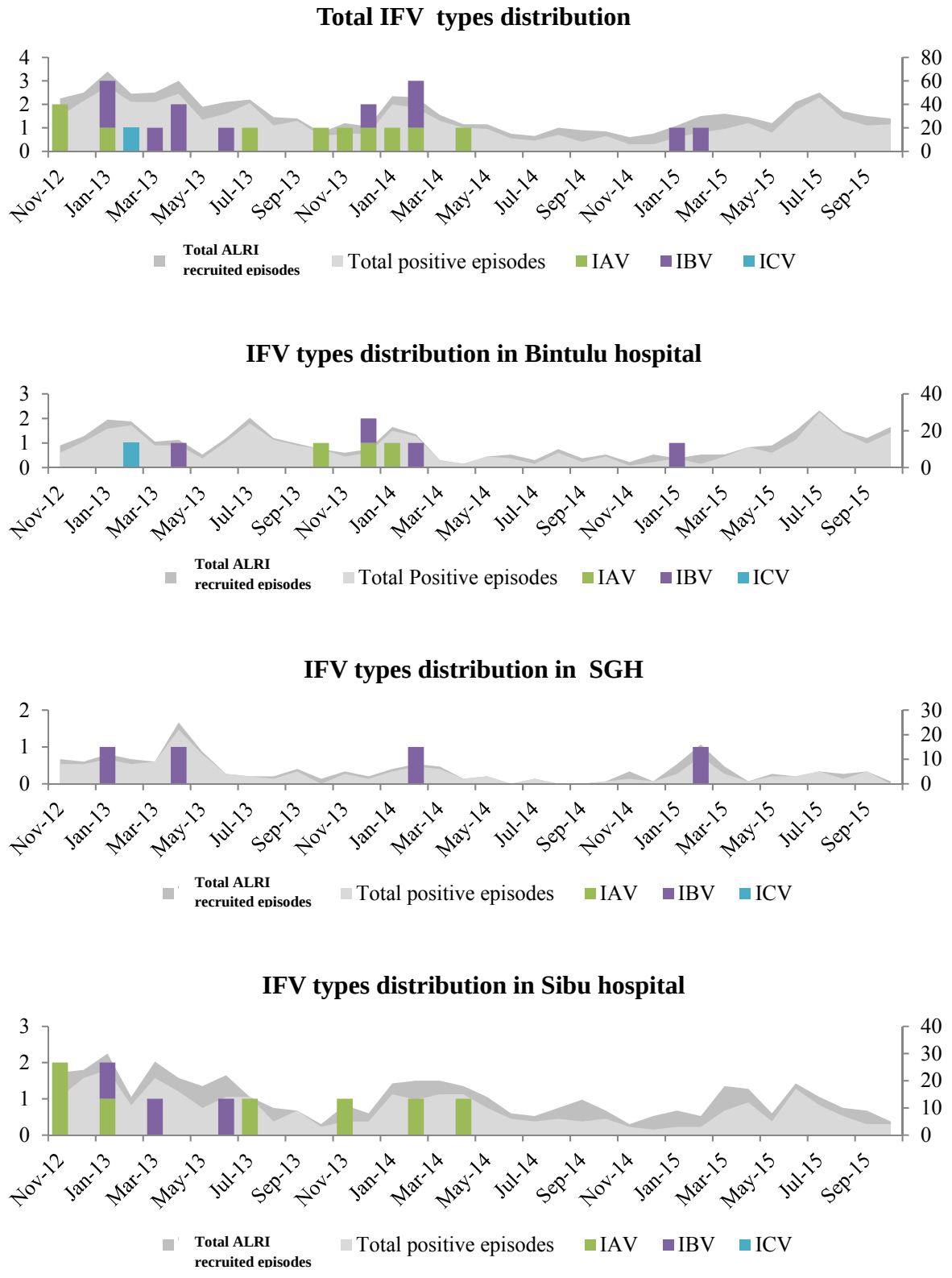


Figure 3.47: Seasonal variations of IFV from Nov 2012- Oct 2013 among children < 5 years old with acute respiratory tract infection.

IFV alone was detected in 54.5% (12/22) of the total IFV positive episodes. The remaining 45.5% (10/22) was co-detected with other respiratory viruses. Of those co-detected viruses, it was observed that 60% (6/10) involved HAdV (Table 3.43).

Table 3.43: Single and co-detection of IFV with other respiratory viruses

Type of detection	**IFV**
Single detection	***12***
Co-detection (Total)	***10***
HAdV	4
HRV/EV	2
HPIV	1
HBoV	1
HAdV + RSV	1
HAdV + HPIV	1

3.11 Negative episodes

The main objective of this study is to describe the viral pathogens detected in ALRI among children admitted to PICU ward. We did not investigate the bacterial causes of ALRI such as pneumococcus, *H. influenza* or *M. catarrhalis*. There were 263 (22.6%) negative episodes documented in this study. It would be reasonable to assume that these negative episodes could possibly be attributed to bacteria, atypical microorganisms or other virus infections other than the 14 respiratory viruses investigated in the present study.

CHAPTER 4

DISCUSSION

4.1 Overview of the epidemiology of severe community-acquired viral lower respiratory tract infection in children aged 5 years and below in Sarawak.

In this study, multiplex reverse transcription-PCR (RT-PCR) and sequencing analysis were used to identify recently recognized viruses such as HMPV, HCoV-NL63, HRV-C and HBoV and to determine the prevalence of other respiratory-associated viruses such as RSV, HAdV, HPIV and IFV. Although routine diagnostic methods for respiratory viruses such as cell culture and immunoassays are useful, multiplex RT-PCR is a more robust method as it offers an enhanced sensitivity combined with rapid detection of a large panel of respiratory viruses (Schreckenberger & McAdam, 2015). Our findings from this 36-month period study in Sarawak demonstrated a high positive detection rate (77%; 902/1165 positive episodes recruited to the study) of respiratory viruses in ALRI among children younger than five years old. By comparison, a similar study by Khor and colleagues in Malaysia demonstrated a 26.4% positive detection rate by using immunofluorescence assays and viral cultures as detection methods (Khor *et al.* 2012).

It was reported that in 2017, pneumonia was the second leading cause of deaths in Malaysia (12.7%) after Ischaemic heart diseases (13.9%) and there was increasing hospitalised pneumonia being seen in Sibu Hospital, thus, ALRI was chosen as disease of interest in this study (Department of Statistics Malaysia, 2018; Toh *et al.*, 2019).

ALRI generally occurs mostly in children under 5 years old. Globally, the disease incidence for severe acute respiratory tract infection was highest in children aged 0–11

months than in children aged 12–59 months (Nair *et al.*, 2010). We observed similar trends in our study where 56.1% (654/1165) cases were of age group 1-12 months. Four age group distribution patterns were shown in Figure 3.3. The age distribution of RSV in this study appears to be similar with other developing countries (Weber *et al.*,1998). The fact that more than half of the RSV detection (51.8%, 277/535) occurred in children under 1 year of age in this study showed that children in this age group are more vulnerable to RSV infections, despite the presence of maternal antibodies. Other respiratory viral studies among children less than 5 years old in Malaysia and Oman also reviewed high percentage of viral detection with 75% and 84% found among those under 1 year of age (Khor *et al.*, 2012; Khamis *et al*. 2012). In general, RSV declined as age increased (Nolan *et al.*, 2015). According to a review by Piedimonte and Perez, nearly all children are infected by RSV at least once by the age of two, but peak incidence occurs between ages 2 and 3 months as a result of limited immunologic protection, smaller airway size, and unique structural and functional features of the developing respiratory tract (Piedimonte & Perez, 2014). In Brazil, Ferone and colleagues found that children with HAdV are older than those with RSV among hospitalized infants (Ferone *et al.*, 2014). In China, a study reported that the incidence of HAdV infection peaked in children aged 3-7 years which revealed that children infected with HAdV were significantly older than children infected with RSV (Jin *et al.*, 2013). This trend was similarly observed in this study, however, the data was not statistically significantly.

Our study showed that RSV (36.1%) was the predominant virus detected in children with ALRI followed by HAdV (28.2%), HRV (16.6%) and HPIV (8.6%) respectively. Globally, RSV was reported to be the leading cause of severe childhood ALRI and the main cause of hospital admissions (Nair *et al.*, 2013). Most studies done in

other tropical and subtropical countries showed that RSV or HRV was the most prevalent virus in children with viral respiratory tract infection (Anders*et al.*, 2015; Do *et al.*, 2011;Hasan*et al.*2015; Tregoning & Schwarze, 2010;Yoshida *et al.*, 2013). Our study showed only 16.6% of HRV mainly due to the reason that most of our patients recruited into this study involved those with pneumonia, whereas HRV were reported to be associated with at least 70% of virus-related wheezing exacerbation, asthma exacerbation, cold and flu-like illnesses (Arden & Mackay, 2010; Mak *et al.*, 2011). In this study, HRV-A and HRV-C were identified as the predominant species in pediatric patients in Borneo which are in accordance with global epidemiologic studies in other parts of the world (Etemadi*et al.*, 2013; Franco *et al.*, 2012; Fry *et al.*, 2011). In 2014, some regions in the USA and Canada experienced a nationwide outbreak of EV-D68 associated with severe respiratory illness which involved mainly children. Our study detected 15 episodes (1.7%, 15/1165) of EV-D68 among all hospitalized episodes during this 3 years study period from November 2012 to October 2015. Over the past 5 years, outbreaks of EV-D68 in Japan, Philippines and the Netherlands as well as in the USA suggested the occurrence of EV-D68 as an emerging respiratory pathogen (Ikeda *et al.*, 2012; Immamura *et al.*, 2011; Meijer *et al.*, 2012; Tokarz *et al.*, 2012).

In previous respiratory studies of this age group, the most common viruses detected were RSV, influenza virus, HPIV, EV and HRV (Khor*et al.*, 2012; Kwofie *et al.*, 2012; Yoshida *et al.*, 2013). Interestingly, however, our study showed a high prevalence of HAdV as the second leading cause of ALRI. HAdV was present in 5.2% and 6% of children aged under 5 years respectively in their study (Khor *et al.*, 2012; Yoshida *et al.*, 2013). However, higher levels of HAdV have been reported in Gabon, Jordan and Brazil with a detection rate of 17.5%, 37% and 25% respectively (Bezerra *et al.*, 2011; Kaplan *et*

al., 2006; Lekana-Douki *et al.*, 2014). Our findings support HAdV as being a highly prevalent virus in paediatric ALRI with a 29.1% detection rate.

During November 2012 to October 2015, we identified HPIV in 8.9% (n=104) of the 1165 ALRI hospital admission episodes in Sarawak, Borneo. Among these, HPIV-3 was the most common followed by HPIV-1. This prevalence is comparable with previous studies which found that HPIV-1 and HPIV-3 are common in China, Japan, Malaysia, Thailand and Brazil while HPIV-2 and HPIV-4 are less frequently detected (Fé *et al.*, 2008; Khor *et al.*, 2012; Mao *et al.*, 2012; Mizuta *et al.*, 2011; Ruampunpong *et al.*, 2014)

Other studies reported that IFV was among the common viral agent detected followed by RSV or HRV. In contrast to those reports, IFV detection accounted for only 1.9% of ALRI among the hospitalized episodes, which was not a predominant cause of pneumonia among hospitalized patients in this study. A study of ALRI by Sentilhes and colleagues in Lao, Cambodia showed that influenza viruses were detected in all age-groups but were more frequent in the age-group above 5 years (Sentilhes *et al.*, 2013). Another epidemiological study of respiratory tract infection among children and adult in Southern China found that the detection rate for IFV was highest among patients aged 15-35 years (Zhang *et al.*, 2014a). Another reason for the low detection rate of IFV might be as suggested by Lu and colleagues that it was more commonly detected in URTIs but was observed at only a low rate in LRTI samples (Lu *et al.*, 2013).

Co-detection of multiple viruses in ALRI have been commonly described in a number of studies (Cebey-López *et al.*, 2015; Goka*et al.*, 2014; Rehder, *et al.*, 2015; Tran, 2013). Among the total positive episodes in our study, the co-detection rate was 30% (270/902) which was similar to 27.9% as reported by Do and colleagues (2011). The three

principal viruses involved in co-detection were RSV, HAdV and HRV. Similar results were reported in recent studies about the prevalent viruses involved in co-detection (Cantais *et al.*, 2014; Do *et al.*, 2011; Kim *et al.*, 2013). However, another study reported HRV, HPIV and IFV as the main viruses involved in co-detection (Lu *et al.*, 2014). Co-detection rate and different groupings of leading viruses involved in co-detection varies widely across countries depending on the panel of respiratory viruses tested, variations in seasonal distributions, geographic location and diagnostic test methods used. RSV-HAdV co-detection was the most frequent in this study followed by HAdV-HRV and RSV-HRV. The overlapping of RSV and HAdV seasons demonstrated in this 3 year study partly explained the high RSV-HAdV co-detection rates. The percentage of co-detection of each virus is determined by the possibility of its seasonal circulation coinciding with that of the other virus as shown in Figure 3.9. The time of year in which viral circulation occurs could enhance the susceptibility of children to viral co-detection. By analysing the co-detection and single detection simultaneously (Figure 3.24) together with the clinical presentation, we are able to hypothesize which virus is likely to be the causative agent as determined by overlap of the same virus detected in episodes of co-detection and single detection. The other co-detected viruses would likely then be bystander/s that is not predominantly responsible as the main causative agent of the clinical presentation observed.

Our study detected only one episode of co-detection between RSV and HMPV although a few studies have found HMPV and RSV co-detection rates of approximately 5-29% (De Paulis *et al.*, 2011; Moattari *et al.*,2015; Xepapadaki *et al.*, 2004). However, there were also studies that reported no virus co-detection between RSV and HMPV similar to the findings of our study (Wang *et al.*, 2014; Essa *et al.*, 2015). A review by Debiaggi and colleagues (2012) found different reports suggesting an association between RSV-HMPV

co-detection and an increase in the disease severity or the absence of an association between dual infection and disease severity. As molecular assays detect only nucleic acid, a positive result does not necessarily mean the presence of the pathogen as the real causative agent. When studying co-detection pattern of respiratory viruses, it's crucial to differentiate the real causative agent of the disease. Further study on viral load and co-detection with disease severity in hospitalized children with LRTI could provide some clue regarding this issue (Martinez-Roig *et al.*, 2015; Franz *et al.*, 2010).

Malaysia is a tropical country located in the Southeast Asia region. This country is separated by the South China Sea into Peninsular Malaysia and Malaysian Borneo. Sarawak is part of Malaysia located on the northwest of Borneo with an equatorial climate. During the northeast monsoon season, the exposed areas like the east coast of Peninsular Malaysia, Western Sarawak and the northeast coast of Sabah experience heavy rain. On the other hand, inland areas are relatively free from its influence. The coastal areas of Sarawak experience a rainfall regime of one maximum and one minimum. The maximum rainfall occurs usually during January whereas the minimum rainfall occurs in June or July. Much of the rainfall is received during the northeast monsoon months of December to March (Malaysia Meteorological Department, 2016).

In our study, respiratory viruses were detected throughout the year. It was found that the distribution of the four main respiratory viruses (RSV, HAdV, HRV, PIV) were similar across the three hospitals recruited to the study in Sarawak. RSV and HAdV are strongly seasonal while seasonality is less clear for HCoV, HBoV, IFV and HMPV which may be due to the smaller number of detections. Despite two of the three RSV epidemics occurring at the end of the rainy season, spanning the dry season, neither rainfall nor temperature was significantly associated with RSV detections in our study. RSV activity

peaked towards the end of the rainy season during the first and second year of our study in 2012-2013 and 2013-2014, however, we observed that RSV epidemic started more gradually with a delayed peak in 2015. Correlation coefficients might underestimate the closeness of a relationship when one of the epidemic seasons lags behind the other (Stewart, 2016).

Previous studies summarized that in tropical or semitropical climates, RSV outbreaks frequently associated with rainy rather than cold season (Sricharoenchai *et al.*, 2016; Stensballe *et al.*, 2003; Weber *et al.*, 1998). In tropics, RSV epidemics appear regularly but with different patterns of seasonality. In northern tropical regions, RSV seems to be associated with a decrease in temperature and an increase in rainfall whereas in southern tropical regions, the seasonality is associated with decrease in both temperature and rainfall. In equatorial regions, RSV appears to be present year round with some increase during the dry months. However, a study performed in Peninsular Malaysia showed that RSV detections was inversely correlated with temperature and were associated with increase in rainy days with an annual peak in November, December and January (Chan *et al.*, 2002). Our neighbouring country, Singapore, in contrast found that RSV detection was associated with higher temperatures but also with a higher temperature fluctuation (Chew *et al.*, 1998). The contradictory patterns of association between RSV and meteorological parameters might due to different geographic distribution, behavioural factors or cyclic changes in a population's immunologic susceptibility (Stensballe *et al.*, 2003; Weber et al, 1998).

In our study, HAdV was found positively associated with temperature while HPIV was positively associated with temperature and negatively associated with rainfall. High temperatures favoured HPIV and HAdV in southeast China (Ji *et al.*, 2011; Chen *et al.*,

2014) which was in accordance to our study. However, there have been published reports that HAdV infections occur throughout the year with no clear seasonal variation (del Valle Mendoza *et al.*, 2015; Alonso *et al.*, 2012; Ampuero *et al.*, 2012) although they have been reported to occur more commonly in temperate countries from late winter to early summer (Tan & Carlos, 2010). In Malaysia, HAdV was present all year-round as well, with a peak in February-March.

Similar to other studies, HPIV circulate at low levels throughout the year (Olsen *et al.*, 2010). The majority of HPIV detected in Fortaleza, Brazil occurred in the dry months as well (Fe *et al.*, 2008). HPIV did not show significant annual seasonality in Singapore or Taiwan (Chew *et al.*, 1998; Tsai *et al.*, 2001). Nevertheless, studies of HPIV infection in Southeast Asia found seasonal peaks: a 2-year study in Thailand found a February-March peak, while in Singapore study found a February-May peak (Chew *et al.*, 1998; Suwanjutha *et al.*, 1990). Our study showed that HPIV occurs mostly during March to September which coincides with the hot months during this 36-months period of study. HRV-A and HRV-C co-circulated in most months while HRV-B occurred sporadically throughout this study which is in agreement with finding shown in other studies (Arakawa *et al.*, 2012; Onyango *et al.*, 2012; Tsatsral *et al.*, 2015). In this study, pneumonia (84.9%, 989/1165) was the most frequent clinical diagnosis observed among children below 5 years of age hospitalized for ALRI followed by acute bronchiolitis (6.8%, 79/1165). The three main viruses involved in pneumonia and acute bronchiolitis were RSV, HAdV and HRV. RSV is the commonest cause of viral pneumonia and bronchiolitis especially in the first 3 years of life. It has been reported that the prevalence of RSV in children with respiratory tract infection to be 14% to 34% and was the main etiologic agent of pneumonia and bronchiolitis globally (Berkley *et al.*, 2010; Kwofie *et al.*, 2012; Lamarão *et al.*, 2012).

We observed a higher prevalence of adenovirus-associated pneumonia (28.2%, 279/989) compared to other studies (Chen *et al.*, 2013; Ruuskanen *et al.*, 2011). This might due to the increase of HAdV in two distinct seasons with HAdV-7 and HAdV-3 as the predominant species in concordance with the worldwide increase of HAdV as in West Malaysia, Singapore, Taiwan and China (Apandi *et al.*, 2012; Ng *et al.*, 2015a; Lai *et al.*, 2013; Tang *et al.*, 2011). It is important to recognize this type of infection as it might induce life-threatening acute respiratory distress syndrome (ARDS) and can be fatal (Alharbi *et al.*, 2012; Lynch *et al.*, 2011).

HRV was detected in 14.9% (149/989) of the children with pneumonia, 26.6% (21/79) among those with acute bronchiolitis, 34.7% (17/49) in acute exacerbation of bronchial asthma, 45% (9/20) among those with newly diagnosed bronchial asthma and 44% (4/9) among those with viral wheeze. Consistent with this, HRV can act as a sole pathogen or in synergy with other pathogens in association with pneumonia (Ruuskanen *et al.*, 2013). In almost all studies of asthmatics and wheezing, the major viruses identified were HRV and RSV. HRV alone are found in 60% of viral asthma exacerbation in hospitalized children and adults (Proud, 2011).

Nine episodes of viral croup were diagnosed in this study. HPIV-1 alone was detected in five and one was found to be co-detected with HPIV-1 and HCoV-229E. It is known that croup in children is mainly caused by HPIV-1. Bronchiolitis and pneumonia are caused by all four types of HPIV, however, HPIV-3 is most frequently detected particularly among young infants and associated with the diagnoses of bronchiolitis and pneumonia which is similar to the findings in our study (Mao *et al.*, 2012).

As an overview, this three-year study provides baseline data of the epidemiology, seasonality, age distribution, co-detection patterns as well as clinical association of viral respiratory infections in hospitalized children with ARTI in Borneo, Sarawak. In this study, RSV, HAdV, HRV and HPIV accounted for 91.5% of paediatric viral ALRIs among children below five years old who were admitted to PICU ward despite the low prevalence of influenza virus. These viruses also contribute to the economic burden of healthcare. Therefore, just like influenza, priority should be focused on RSV, HAdV and HRV as well. Accurate and rapid diagnosis of these viruses can help physicians optimize patient treatment and outcomes. There were overlapping seasonal trends observed between RSV and HAdV throughout these 3 years of study. HAdV and HPIV were positively associated with temperature which detected mainly during the hot months. Pneumonia remains a leading cause of ALRIs in Malaysia and showed high prevalence in Sarawak (Department of Statistics Malaysia, 2017; Toh *et al.*, 2019). From a public health perspective, information on the prevalence and seasonality of respiratory viruses are crucial to be used as a basis to optimize the timing of interventions for future vaccination programs such as RSV vaccines.

4.2 Respiratory Syncytial Virus (RSV)

RSV is one of the most important respiratory pathogens among infants worldwide. The identification of RSV in more than one third of the total hospital admission episodes of which 67% of it was single infections in this study confirmed that this virus is a dominant agent of respiratory disease in children. This finding is in line with previous reports from our local studies in Malaysia (Chan *et al.*, 1999; Etemadi *et al.*, 2013; Khor *et al.*, 2013;

Rahman *et al.*, 2013; Zamberi *et al.*, 2003) and also other countries worldwide (Anders, *et al.*, 2015; Fattouh *et al.* 2011; Johani & Akhter, 2011; Tatochenko *et al.*, 2010).

RSV has been referred in the literature as the main etiology responsible for bronchiolitis and pneumonia which mostly infect children aged 2 years or younger. The frequency of RSV decreased with increasing age (Barberi *et al.*, 2012; Gurgel *et al.*, 2016; Medici *et al.*, 2004). In our study, of the 434 RSV positive results, 87.8% of RSV were detected in children younger than two years old, among which 63.8% were aged ⩽ 12 months, suggesting that illnesses caused by RSV can be severe in this age group, thus requiring hospitalization with prompt and effective medical intervention (Lee *et al.*, 2007b; Zhang *et al.*, 2014b). Various studies(Fry *et al.*, 2010; Mishra *et al.*, 2016; Shi *et al.*, 2017; Sung *et al.*, 2011) have demonstrated that RSV infection is most prevalent in the first year of an infant's life which is in concordance with the result of our study.

The ratio of male to female in this study is 1.5:1. The majority of those in which RSV was detected were male. Several studies reviewed the male sex as a risk factor for severe RSV LRTI (Oliveira *et al.*, 2008; Simoes, 2003; Zhang *et al.*, 2014b). The reason might be due to the anatomic nature that boys have shorter and narrower airways and thus are more likely to develop respiratory illness in the case of RSV infection (Sommer *et al.*, 2011).

This study which covered three consecutive RSV seasonality, from November 2012 to October 2015 is the first observational study aimed at defining the epidemiology pattern of RSV infection in young children aged ⩽ 5 years in Sarawak. The RSV season observed in this study varied substantially by year with the peak RSV activity occurring in January – February during the 2012-2013 and 2013-2014 season but in June-July during the 2014-

2015 season. Considering the variability of epidemiological studies conducted worldwide, the late arrival of RSV peak was not exceptional. Surveillance studies from other countries have identified RSV outbreaks pattern with early and late onset of seasonal outbreak where later seasons are a regular occurrence not associated with the weather (Medici *et al.*, 2004; Stensballe *et al.*, 2003; Terletskaia-Ladwig *et al.*, 2005).

A consistent seasonal variation was observed with RSV in a retrospective study carried out by Chew and colleagues (1998) from 1990 to 1994 in Singapore, with annual recurring peaks of RSV occurring from March to August. These peaks were associated with higher environmental temperature, lower relative humidity and higher maximal day-to-day temperature variation. However, no association with rainfall was found (Chew *et al.*, 1998). In contrast to another study done in Peninsular Malaysia from 1982 to 1997 among children under 24 months of age hospitalized with LRTI, a clear seasonal trend for RSV were observed with annual peaks occurring between November and January which coincided with the rainy season in Malaysia (Chan *et al.*, 2002). Also in another 6 month local study by Etemadi and colleagues (2013) at Hospital Serdang, Malaysia, RSV was seen in July and then started to increase from August to October. Data from our study suggests three distinct waves of RSV outbreak with a peak in January-February during the first two seasons but in June-July during the last season of the study. This further suggested that in tropical regions with distinct yearly outbreaks, the timing of outbreaks can vary more year to year than in temperate climates. In our study, there was no significant association between RSV with meteorological factors. This might due to the lag effect of RSV which occurred during the late seasonal outbreak in 2014-2015. Nevertheless, we did observe that RSV peak in January-February during the first two seasons which coincide with the rainy seasons in Malaysia.

Both the prevalence of RSV and the peak of RSV activity were similar in the three participating hospitals. In further analysing the RSV positive results to determine the RSV group in the three participating hospitals, all the hospitals involved in this study showed that RSV A is more prevalent compared to RSV B. Among the three districts, RSV A was detected close to 60% while RSV B detected near to 40%. However, RSV-B was dominant in the first epidemic season (2012-2013) and there is a shift into RSV-A predominance in the next two epidemic seasons. These findings are in agreement with majority of studies by other countries around the world including Kenya, Japan, India, China and Vietnam with various patterns of subgroup predominance (Parveen *et al.*, 2006; Sato *et al.*, 2005; Tran *et al.*, 2013; Zhang *et al.*, 2007). Locally, Khor and colleagues conducted a study from 1989 to 2011 in Kuala Lumpur, Malaysia and showed that both RSV subgroups were also found co-circulating each year with RSV-A dominant in 12 out of 17 years (Khor *et al.*, 2012).

Decreased herd immunity in the population especially among mothers might affect the peak of spread which leads to increased susceptibility in their newborns. During the latter part of the epidemic, maternal antibody concentrations are high due to immune response with RSV infection, thus infants born in the latter part of the epidemic would be protected, leading to a decreased in susceptible infants to RSV disease. Babies conceived at the end of RSV season may have lower antibody concentrations due to a natural decline in maternal antibodies; when these babies are born 9 to 10 months later, they would be susceptible to RSV (Stensballe *et al.*, 2003). Different spread of the RSV or the genomic variants may also lead to differences in the epidemics. Certain RSV genomic types may appear predominantly each year while some types might present in consecutive epidemics

or some being replaced by emerging types (Johansen *et al.*, 1997; Reese & Marchette, 1991).

In this study, almost one-third (32.7%) of RSV co-detection was reported among the positive episodes of ALRI caused by this virus. Although they were mainly dual-detections, with 3 or more viruses also detected simultaneously. The most frequent co-detection observed was between RSV and HAdV, and the second was between RSV and HRV. Nevertheless, co-detections with all the other respiratory viruses were detected as well. Studies focusing on the relative importance of respiratory viruses co-detection generally have resulted in equally divergent findings. Some studies documented an increase in clinical severity in children with more than one virus detected (Aberle *et al.*, 2005; Calvo *et al.*, 2008; Cilla *et al.*, 2008), while others reported that infection with RSV alone was more severe than multiple virus infection (Canducci *et al.*, 2008) and certain studies found no association of co-detection with clinical severity (Calvo *et al.*, 2015a; De Paulis *et al.*, 2011; Nascimento *et al.*, 2010; Suryadevara *et al.*, 2011). Possible explanations for these discrepancy might be due to variation in age groups, the extent of disease severity, the proportion with underlying conditions and their adjustment in multivariate analyses (Asner *et al.*, 2015).

As in some studies, RSV-HAdV was the most common co-detected combination found. As reported by De Paulis *et al.* (2011) who studied viral co-detection in infants hospitalized with acute lower respiratory tract infections, the most frequent virus associated with RSV co-detection was HAdV (43.6%). Stempel *et al.* (2009) also reported of the 139 children detected with RSV using FA or real-time PCR, 34 children (24%) had multiple viruses identified and RSV were co-detected mostly with HAdV (17, 50%). However, both the authors did not look for HRV detection in their study, thus the co-

detection rate may be underestimated. In another study by Calvo *et al.* (2015a), RSV-HRV was the most frequent co-detection found. They also reported that viral co-detection showed mixed clinical features without presenting any greater severity. Similarly, in a study which evaluate the role of HRV infections by Costa *et al.* (2014b), they found that HRV single infections were less severe than co-infections with RSV. HAdV have been frequently detected during this study period and appeared to be the second predominant agent of single virus detection in hospitalized children with acute respiratory disease. The high prevalence of viral co-detection of RSV with HAdV may be due to the reasons that our study populations mainly composed of infants less than one year old who were hospitalized with pneumonia and bronchiolitis. In addition, co-detection of RSV and HAdV in our study had a seasonality that matches with the RSV circulation during January–March 2013 and June-July 2015 confirming the importance of RSV in this association.

In conclusion, to our knowledge, this is the first report of RSV detection in hospitalized children with severe ALRI in our Sarawak region. We highlighted that RSV prevalence in Sarawak is similar to those found in other regions and countries worldwide. The data provided in this study confirmed that RSV still represents a large burden of disease among those hospitalized infants with severe acute respiratory diseases. RSV-A was identified as the predominant species during this study period. There was a variation in RSV season and thus the activity of RSV need to be monitored every year to define the period with high risk of infections. Local data is essential to more accurately predict the RSV outbreak seasons in a given community. Mixed RSV-HAdV should be taken into account when planning intervention in order to reduce the burden of acute lower respiratory tract infections. Further studies are needed to evaluate the genotypes

responsible for the disease severity besides to understand the association of RSV in relation to clinical features. If we are to manage LRTI in hospitalized children more efficiently, we need to broaden our knowledge on the epidemiological aspects of RSV and respiratory diseases.

4.3 Human adenovirus (HAdV)

It is known that HAdV is among one of the major causative agent for acute respiratory illness among young children (Chen *et al.*, 2016). In developing countries such as Malaysia, Vietnam and China, HAdV accounted for about 4-10% of hospitalized children with viral ALRI (Buecher *et al.*, 2010; Liu *et al.*, 2014; Yoshida *et al.*, 2010). For the purpose of this study, a published adenovirus nested RT-PCR was optimized for better HAdV detection with higher sensitivity (Cardosa *et al.* 1999). As a result, we detected HAdV as the second most frequent virus among hospitalized children who required intensive care due to ALRI with a higher positive rate of 29.2%.

Similar with other respiratory viruses detected in our study, children younger than two years of age were the most susceptible to respiratory tract infections of HAdV (83.5% of total HAdV positive episodes). This was in line with previous reports that most HAdV was detected among children aged < 2 years (Calvo *et al.*, 2015b; Ng *et al.*, 2015; Qurei *et al.*, 2012). The male to female ratio in our study is 1.6. The tendency of male predominance is reported in most of the respiratory studies involving HAdV and also other respiratory viruses such as RSV, HPIV and IFV (Ampuero *et al.*, 2012; Lai *et al.*, 2013; Mandelboim *et al.*, 2011; Mathisen *et al.*, 2009; Nair *et al.*, 2013). However, the reasons for this have not yet been elucidated.

One of the specific objective of this study was to characterize the molecular types of main respiratory viruses which included HAdV as the second most prevalent virus detected during the study period. We optimized the multiplex PCR followed by sequencing of partial hexon gene which allowed us to identify the molecular types of HAdV that have not previously been described in Sarawak. Our study found that HAdV species B and C are the major circulating strains in children with severe ALRI admitted to PICU and represented 71.2% and 25.3% respectively the positive HAdV episodes in this study. In our study, the most common molecular types identified include HAdV-7, HAdV-3, HAdV-2 and HAdV-1, which were similar to other published reports(Lee *et al.*,2010c; Mandelboim *et al.*, 2011; Palomino *et al.*, 2004). Additionally, we also identified some HAdV molecular types that were detected in small numbers that were not routinely reported in Malaysia and these include HAdV-8, -36 and -54 of species D, HAdV-4 of species E, and HAdV-40 and -41 of species F. The species F type 40 and 41 detected are commonly associated with acute diarrheal disease across the world.

In Malaysia, there are limited studies describing the epidemiological findings associated with HAdV. Recently, a study which involved patients who sought treatment for respiratory illness at government hospitals in Malaysia from 2003 to 2011 by Apandi *et al.*, (2013) found that 64% of ARI cases were of HAdV species C and 36% were of HAdV species B. Another study by Abd-Jamil *et al.*, (2010) also reported that 70% of the HAdV identified from pediatric patients who sought treatment for respiratory tract infection in UMMC, Malaysia from 1999 to 2005 was of species C. This differed with the findings of our study in which we found HAdV species B predominated over HAdV species C. HAdV-7 was the most predominant in 2012-2013 season and HAdV-3 was more prevalent in 2015 during the two peak season. The differences observed between their study and ours

may be due to the following reasons. In their studies, they do not focus on severe ALRI, both inpatient and outpatient children were included, not just hospitalized children. This factor may account for the type distribution difference between their studies and ours. Furthermore, it was reported that children with HAdV-B were more likely than those with HAdV-C to have an acute inflammatory response (leukocytosis, band forms, and elevated CRP), and to be hospitalized for lower respiratory tract infections and gastrointestinal distress(Ya-fang Wang *et al.*, 2016). Infections with HAdV-C often are endemic, mild and most commonly seen in young children (Lee *et al.*, 2010a). Compared with HAdV-B infected patients, they were more frequently admitted to the ICU and mechanically ventilated (Scott *et al.*, 2016). Our findings of severe infection with HAdV-B especially of type 7 requiring ICU admission and hospitalization is consistent with other reports linking HAdV-B7 with more severe acute respiratory disease, hence we reported a higher detection rate of HAdV-B than HAdV-C (Carballal *et al.*, 2002; Cui *et al.*, 2015; Mitchell *et al.*, 2000).

It is important to note that our study mainly involved children with severe ALRI who were admitted to PICU. Our finding is consistent with other studies that HAdV-B causes more severe respiratory tract infections than other molecular type and is more likely to result in hospitalization (Wang *et al.*, 2016). Although HAdV-1-3, 5, and 7 are frequently detected from children with ARTI, outbreaks of severe infections are mostly reported with HADV-7, followed by HAdV-3 (Callaway *et al.*, 2011; Lai *et al.*, 2013; Tang *et al.*, 2011). Several adenovirus outbreaks have been reported in Asia. An outbreak of acute respiratory disease was reported at the Kuala Lumpur Police Training Centre in March-April 2011 in which HAdV-7 was identified as the etiologic agent (Apandi *et al.*, 2012). In addition, there was a marked increase in severe HAdV-7 in pediatric inpatients at

Singapore during November 2012-July 2013 which occurred at a similar period of time with the HAdV-7 outbreak in our study during December 2012-August 2013 (Ng *et al.*, 2015a) Researchers from Taiwan reported several outbreaks of HAdV involving HAdV-3, -7 and -4 between 1999 and 2001 (Lin *et al.*, 2004), another in 2004-2005 with HAdV-3 (Chang *et al.*, 2008) and in 2011 with HAdV-3 and -7 causing acute respiratory failure in children (Lai *et al.*, 2013). HAdV-3 and 7 are also two of the most important etiological agents of pneumonia in China. It was reported that HAdV-7 contributed to an outbreak of pneumonia in infants in 2011 and showed a higher fatality rate compared to HAdV-3 which was similar with the findings by Lai and colleagues in Taiwan (Tang *et al.*, 2011).

Our study also revealed the seasonal pattern of HAdV associated with hospitalized children in Sarawak. There is very little published data on HAdV circulation in the tropics, therefore, the overall pattern of seasonality is limited and scarce. In Malaysia, one study reported that HAdV was present all year-round with a peak in February – March (Khor *et al.*, 2012). Another study done recently in Seremban, Malaysia also found an increase of HAdV in 2013 between March and June with a small peak between September and December. However, serotyping for HAdV was not done in their study due to limitation of PCR methods available in the hospital laboratory (Ng *et al.*, 2015a). As previously mentioned in the literature review chapter, HAdV infections occur throughout the year without clear seasonal variation (Ampuero *et al.*, 2012; del Valle Mendoza *et al.*, 2015). Although HAdV was generally detected throughout the year, our study revealed major peaks occurred biennially during July of each of the epidemic season. Furthermore, we found that HAdV detection was positively correlated with mean temperature but negatively associated with rainfall. This finding is similar with a four years study of hospitalized

children with ARI in Suzhou, China which found that HAdV detection was most frequent during warm, dry months being positively correlated with temperature (Ji *et al.*, 2011).

The co-detection pattern was also investigated in this study as well. A total of 58.2% of the HAdV positive episodes were co-detected with other respiratory viruses where RSV and HRV represented the most common co-detected viruses with HAdV. The high co-detection rate may be due to infants immature immune system and the lack of previous exposure to respiratory viruses which tend to increase their susceptibility to multiple virus infections (Simon *et al.*, 2015). It has been reported that some viruses such as HAdV, HBoV and HMPV were mainly present as co-pathogen, as observed by other authors (Bezerra *et al.*, 2011; Calvo *et al.*, 2015b; Choi *et al.*, 2006). Another possible explanation for the high prevalence of HAdV co-detection could be due to asymptomatic persistence and prolonged virus shedding (Kalu *et al.*, 2010). The association between co-detection and clinical manifestation is not investigated in this study. Further study is needed to compare the severity between single HAdV detection and HAdV-RSV co-detection. However, it was reported in another study that the severity in the group with single HAdV detection and HAdV-RSV co-detection was the same (Palomino *et al.*, 2004). Finding by Asner *et al.* (2015) also support equivalent disease severity between single virus infection and virus coinfection. HAdV is unique among the common respiratory viruses whereby it can spread to other organs resulting in gastroenteritis, conjunctivitis and meningoencephalitis. It was reported that children infected with HAdV-7 had hepatomegaly or elevated hepatic aminotransferase levels which is suggestive of hepatic involvement (Hong *et al.*, 2001).

In summary, this study enabled us to have an overview of the common HAdV molecular types in hospitalized patients in Sarawak from November 2012 to October 2015.

Additional studies on clinical manifestations are needed to determine the role of HAdV co-detection in the severity of disease with other respiratory viruses. More attention should be paid to the difference in circulating types and seasonal peaks of HAdV between Peninsular Malaysia and East Malaysia and disease severity. The observation that different subtypes especially HAdV-7 and HAdV-3 in those children who admitted to PICU with ALRI particularly pneumonia and bronchiolitis is particularly important in the future for more effective management of hospitalized children with LRTI. Extensive and continuous surveillance for HAdV molecular epidemiology is necessary and may assist in the future when considering possible vaccination of the populations.

4.4 Human rhinovirus (HRV)

This is the first study that characterizes the type of HRV in children less than five years old who were admitted to PICU ward in Sarawak, Borneo. In this study, HRV was the third leading cause of severe ALRI accounting for 15.5% (181/1165) of the total hospital admission episodes which indicate that HRV contribute substantially to ALRI requiring hospitalization. This is similar to other pediatric respiratory studies which had proportions that varied between 7.7% and 37.6% as reported previously (Arakawa *et al.*, 2012; Daleno *et al.*, 2013; Fry *et al.*, 2011; Huang *et al.*, 2009; L'Huillier *et al.*, 2015). Our data showed that most of the HRV detection corresponds to HRV-A (48.6%) and HRV-C (47.5%) whilst HRV-B was sporadically detected representing 3.9% of the HRV positive results. This result is similar with findings from other studies done in various countries which showed that HRV-A and HRV-C were most frequently detected whereas HRV-B was less commonly detected (Arakawa *et al.*, 2012; Daleno *et al.*, 2013; Onyango *et al.*, 2012; Tsatsral *et al.*, 2015).

HRV was principally detected among children under 2 years old with ALRI as has been reported in other studies (Daleno *et al.*, 2013; Landa-Cardeña *et al.*, 2012; Piotrowska *et al.*, 2009). Interestingly, the majority of the HRV-A were found in children 1-12 months of age; while those detected with HRV-C were mostly 13-24 months. This finding was similar to a study done recently in Malaysia by Etemadi *et al.* (2013). In another study by Fry *et al.*, (2011) in Thailand, it was found that HRV-A was also the most commonly detected species among hospitalized infants < 1 year of age but HRV-C more commonly detected among children aged 1-18 years.

In this study, the most common diagnosis associated with HRV was viral pneumonia (73.5%) followed by acute bronchiolitis (9.9%) and acute exacerbation of bronchial asthma (8.8%). This is consistent with other studies which stated that HRV is frequently recognized and associated with pneumonia and bronchiolitis in infants and young children even in developing countries which suggest that HRV is an important causative agent of pneumonia and ALRI. Besides that, infection with HRV can cause exacerbation of preexisting airway diseases such as asthma (Jin *et al.*, 2009; Ruuskanen *et al.*, 2013).

The detection of HRV by using nasopharyngeal specimen or nasal swab had been controversial since it can represent upper respiratory tract infection or be due to the prolonged shedding of the virus in previous infection and it had been reported that HRV can be identified in 12-35% of asymptomatic subjects (Fry *et al.*, 2011; Ruuskanen *et al.*, 2013; Wright *et al.*, 2007). It would be valuable if future work includes the prospective collection of asymptomatic nasopharyngeal viral infections in a healthy control group which might help to clarify HRV as a causal agent or just a bystander. However, it is important to mention that all of the patients recruited into this study are hospitalized in

PICU ward with severe ALRI. Additionally, in our study, we have detected HRV as a single agent in 45.9% of the children in which this virus was identified. Furthermore, a study by Piotrowska *et al.* (2009), supported the recent findings of HRV as a major cause of morbidity among young children and suggested that the detection of these viruses at least in children less than 2 years old does not simply represent asymptomatic infection. In addition, according to Louie *et al.* (2009), HRV may be associated with more severe LRTI in children than previously reported, especially in the non-influenza and non-RSV season.

More than half (54.1%) of the hospitalized children with HRV detection had co-detection with other respiratory viruses. We observed 45.3% (82/181) in dual detection, 8.3% (15/181) triple detection and 0.6% (1/181) quadruple detection among children with HRV. The most frequent co-detection appeared between HRV and HAdV which accounted for 25.4% followed by HRV and RSV in 13.3%. This is similar with findings of other studies whereby HRV has been found in dual detection with other respiratory virus in 26% to 58% of cases (Aponte *et al.*, 2015;Fry *et al.*, 2011; Lauinger *et al.*, 2013; Xiang *et al.*, 2010). Similar to our results, studies from Kuwait and Korea also demonstrated that the leading combination in co-detection involved HRV and HAdV for those with pneumonia and bronchiolitis (Essa *et al.*, 2015; Kim *et al.*, 2013). Although RSV was the most prevalent virus detected throughout this study, more HRV was co-detected with HAdV. To further explain, most of the co-detection between HRV and HAdV were detected during HAdV epidemic season in 2015 which have been explained in the previous chapter. In other studies from various countries showed that HRV most frequently co-detected with RSV in children with pneumonia which may be related to the seasonal trend of respiratory viruses (Calvo *et al.*, 2015a;Fry *et al.*, 2011; Paula *et al.*, 2011; Xiang *et al.*, 2010). However, the

clinical relevance of co-detection with the clinical illness is still uncertain (Ruuskanen *et al.*, 2013).

In general, HRV are more commonly detected during the spring, summer and fall months in temperate countries whereas in tropics, there is a possible peak incidence during the rainy season (Jacobs *et al.*, 2013). However, in our study, HRV were detected almost every month throughout the three years. There was neither a clear seasonality nor significant influence of weather (rainfall and temperature) on HRV occurrence in Sarawak. Nevertheless, there was actually limited data available in tropics regarding the association of meteorological factors with HRV. Certain researchers had reported also that HRV does not show clear seasonal pattern in Kilifi, Mongolia and Japan (Arakawa *et al.*, 2012; Onyango *et al.*, 2012; Tsatsral *et al.*, 2015).

Phylogenetic analysis on the VP4/VP2 gene which is predictive of the HRV species and type is now widely used in epidemiological studies (McIntyre *et al.*, 2013). Results from previous studies suggested that most or all HRV types circulate freely worldwide. In this study, we detected a total of 77 genotypes (38 HRV-A, 2 HRV-B and 37 HRV-C). Our data showed a large number of genotypes circulating during each season, and some can circulate in more than one year and simultaneously in different geographic areas. Considering HRV-A12 and HRV-Cpat_10, both were found circulating simultaneously in 2013 at the three different hospitals located in different region in Sarawak. Furthermore, HRV-A12 was the most prevalent HRV-A circulated worldwide besides the most common genotype found in children with a primary diagnosis of viral pneumonia in Beijing, China which was similar with our study (Daleno *et al.*, 2013; McIntyre *et al.*, 2013). We found a high prevalence of HRV-Cpat_10 (EU590054, which was currently assigned as HRV-C53) in this study. Our study showed a higher diversity in HRV circulation in species A and C

compared to other studies done in Peninsular Malaysia. Comparison with HRV-A and C from Peninsular Malaysia detected between 2007 and 2011 also showed the presence of HRV A-12, HRV-A21, HRV-A24, HRV-A39, HRV-A41, HRV-A43, HRV-A46, HRV-A47, HRV-A55, HRV-A56, HRV-A101, HRV-C2, HRV-C6, HRV C-10, HRV-C12, HRV-C14, HRV-C17, HRV-C22 and HRV-C-42 (Chan *et al.*, 2012; Etemadi *et al.*, 2013). Although HRV-B and HRV-C clustered with known genotypes, there were a number of clusters for HRV-A that were not so closely associated with the known genotypes. However, in the absence of the VP1 sequence, the putative new types are considered provisionally assigned.

We conclude that HRV is an important pathogen among young children. As HRV appeared to be more prevalent than certain other common respiratory viruses such as influenza viruses among children seeking medical care, the clinical significance of HRV may have been underestimated. HRV-A and HRV-C were predominantly found in our study with both displaying a high genetic variation in Sarawak which concurs with the global epidemiologic studies worldwide. Several different genotypes of HRV were detected including outbreak-like clusters such as HRV-A12 and HRV-Cpat_10. Future studies will include corroboration of the clinical significance by investigation into the laboratory findings to better explore and understand the role of HRV in ALRTI.

4.5 Human parainfluenza virus (HPIV)

In the following sections, we discuss the prevalence of HPIV, flu virus and also recently identified viruses (EV, HMPV, HCoV and HBoV) detected by RT-PCR during three year study period from November 2012-October 2015. Of the viruses investigated in this study, HPIV was the fourth most frequently detected virus. The prevalence of HPIV-3 was

substantially higher than HPIV-1 and HPIV-4, which is consistent with findings of other studies (Aguilar *et al.*, 2000; Lee *et al.*, 2010c; Pecchini *et al.*, 2015;Wang *et al.*, 2015). It is generally observed that HPIV-3 is the most frequently detected HPIV type in hospitalized patients, especially among young infants, and is often associated with the diagnoses of pneumonia and bronchiolitis, similar to the observations in our study (Fé *et al.*, 2008; Hall, 2001; Henrickson, 2003). More importantly, our study showed that HPIV-1 was mainly detected from patients with viral croup. Evidence from other study confirmed that HPIV-1 was associated with the diagnosis of croup (Vega-briceño *et al.*, 2007). HPIV-4 was detected less often in patients, perhaps because it causes less severe disease.

In this study, HPIV-1 was mainly detected in 2013 and 2015 while HPIV-3 was detected all year-round for samples from Bintulu hospital. As for SGH and Sibu hospital, it was observed that HPIV-3 was instead mainly detected in 2013 and 2015 while HPIV-1 was found predominantly during 2014. This suggests that HPIV may have both endemic and epidemic patterns, and the seasonality of HPIV varies depending on regions. In this study, HPIV-3 was mainly detected in March to September. A study by Khor *et al.* (2012) in Peninsular Malaysia showed that HPIV-1 and HPIV-3 activity was mainly seen in March-May which was similar with our study. Furthermore, in Thailand, HPIV peak activity was found in February-March, while in Singapore, they found a February-May peak (Suwanjutha *et al.*, 1990; Chew *et al.*, 1998). Besides that, it was observed that the frequencies of HPIV-3 increase when detection of HPIV-1 and HPIV-4 detection declined. Earlier studies support these findings (Fry *et al.*, 2006; Glezen *et al.*, 1984; Knott *et al.*, 1994). Possible explanations for this might due to non-specific viral interference or heterotypic cross-protection of antibodies (Welsh *et al.*, 2011). HPIV-1 and HPIV-3 share several common antigens and cross reaction between both antisera has been reported,

especially during reinfection with a heterotypic virus (Henrickson, 2003). Thus, it is possible that prior infection might infer some protection against infection from the other serotype during that season.

4.6 Enteroviruses

Our study demonstrated the presence of EV-D68 in Sarawak since 2013 among patients with severe acute lower respiratory tract infections. Although the overall prevalence of EV-D68 appears to be low at 1.3% of positive episodes in this study, those sporadically detected EV-D68 has been recognized as a re-emerging respiratory virus in several countries worldwide (Jacobson *et al.*, 2012; Steven*et al.*, 2004). Furthermore, EV-D68 deserves more attention in recent years, especially in 2014 due to the nationwide outbreak in relation with severe respiratory illness in the USA.

Similar to other enteroviruses, the EV-D68 VP1 gene has been used to distinguish various enterovirus serotypes which involved mostly in viral binding to host receptors as well as in host immune recognition (Oberste *et al.*, 1999). Phylogenetic analyses of all known EV-D68 revealed that these viruses have emerged into three major evolutionary clades which are distinguishable by nucleotide substitutions and deletions relative to the Fermon strain (Tokarz *et al.*, 2012). Our phylogenetic study was based on VP4/VP2 region. Nevertheless, a study done by Imamura *et al.* (2013) showed that sequences belonging to lineage 2 in the phylogenetic tree based on VP1 sequences were classified into the same cluster in the phylogenetic tree for VP4/VP2 region. This suggested that VP4/VP2 gene used for phylogenetic analysis may represent also the different clades identified. Results obtained in the present study suggested EV-D68 could be divided into 2 clusters based on

their phylogenetic relationship. Many clade B cases had been reported in Europe and Asia including China and Thailand prior to 2014 (Meijer *et al.*, 2012; Poelman *et al.*, 2015; Thongpan *et al.*, 2015; Xiao *et al.*, 2015). Similar with Chongqing, China, clade A and clade B were the main epidemic genotypes reported in 2009 to 2012 (Xiao *et al.*, 2015). Through various studies, it was suggested that the predominant clade might shift from clades A and C to clade B, and clade B might expand worldwide (Du *et al.*, 2015).

After 2014 US outbreak, novel clade and subclades B1 to B3 and D have been identified (Dyrdak *et al.*, 2016; Gong *et al.*, 2016; Yip *et al.* 2017; Wang *et al.* 2017). We observed 60% of the viruses clustered within the new subclade B3 with high homologies to isolates from China, Taiwan & Vietnam while 40% clustered in new clade D similar to isolates from Germany & China. Most of the EV-D68 strains detected in US 2014 outbreak belonged to clade B1 (Midley *et al.*, 2014). Similar to our study, EV-D68 subclade B3 was the predominant lineage circulating in Hong Kong, China and Taiwan since 2014. No EV-D68 subclade B3 had been identified in US until 2016 (Xiao *et al.*, 2015, Gong *et al.*, 2016). Available data suggested that the B3 lineage arose recently in the evolution of EV-D68 and is actively spreading in parts of Europe and US during the 2016 season may have originated from strains that emerged in Asia in 2014 (Du *et al.*, 2015). Furthermore, the previously recognized subclade A2 strains should re-designated within clade D (Gong *et al.*, 2016; Yip *et al.*, 2017). Clade D strain has been circulating globally for example in China, France and Italy (Gong *et al.*, 2016). According to the study done by Ng *et al.*, (2015b), the newly sequenced strains from Peninsular Malaysia were found within clade A (should reclassified as clade D) and clade B which is similar with our study.

In addition, phylogenetic analysis of VP1 sequences suggested that the major EV-D68 epidemic clade varied across time due to rapid evolution of enteroviruses. Further

investigation into VP1 sequences and full genome analysis on available isolates may help to identify if there is any amino acid mutations in the VP1 protein or the 5'UTR which might change the virus behaviour besides to identify and compare the EV-D68 strain in Sarawak with other countries worldwide.

4.7 Human metapneumovirus (HMPV)

The overall HMPV detection among children less than five years old hospitalized with ALRI in Sarawak, Borneo between November 2012 and October 2015 was 5.7% of total positive episodes. However, the incidence of HMPV detection varied annually. The prevalence of HMPV which caused ARTI in children varied from 2 to 15% according to previous studies (Edwards *et al.*, 2013; Esper *et al.*, 2003; Freymuth *et al.*, 2003; Garcia- *et al.*, 2006; Pilger *et al.*, 2011; Williams *et al.*, 2010). Cambodia reported an overall incidence of HMPV to be 1.7% among hospitalised patients with ALRI (Arnott *et al.*, 2013) whereas Bastien and colleagues observed the HMPV incidence rate amongst Canadian patients of all age groups to be 14.8% (Bastien *et al.*, 2003). Our neighbouring country, Singapore reported an incidence rate of 5.3% (Loo *et al.*, 2007). In Malaysia, 53 out of 1580 (3.4%) respiratory samples received by Institute of Medical Research from January 2012 till December 2012 were found to be HMPV positive (Vinomarlini *et al.*, 2014). Another study which described the molecular epidemiology of HMPV in children with ARTI at University of Malaya Medical Centre reported the prevalence rate of HMPV between 2010 and 2012 was 1.1% (Nor'e *et al.*, 2014). The differences might be due to the study population (age, disease severity, patient recruitment criteria involving in-patient and/or out-patient). In addition, certain studies investigated the presence of HMPV only

among those samples that are previously negative for other viruses (Freymuth *et al.*, 2003; Vicente *et al.*, 2003). Furthermore, using frozen samples might also diminish the virus yield.

As mentioned earlier in the result chapter, the annual prevalence of HMPV detection in this study varied from 5.6% in 2012-2013 season to 4.4% in 2013-2014 season and decreased to 2.6% in 2014-2015 season. The annual seasonal peak of HMPV has been observed to vary. Previous studies had described that HMPV can be detected year round, but this virus typically peak in the late winter or spring in temperate countries and during the rainy season in most tropical countries, coincident with or slightly later than RSV season (Arnott *et al.*, 2013; Chow *et al.*, 2016; Mizuta *et al.*, 2013). In our study, we observed the same in which HMPV generally peaked after the RSV season. However, we were unable to comment on the seasonality of HMPV due to the low number of HMPV detected during 2013-2014 season and 2014-2015 season. In contrast to other studies in peninsular Malaysia which reported that the peak of HMPV infection occur during the rainy season, our study however showed an increase during the dry season between April and September (Chow *et al.*, 2016; Vinomarlini *et al.*, 2014). Nevertheless, there is no significant correlation between meteorological factors and HMPV detection rates in this study. Furthermore, consistent with the findings of Oliveira *et al.* (2009), HMPV circulated throughout the year with a small number of sporadic cases during the 36 months study period.

In this present study, Bintulu hospital showed a higher prevalence compared to other two study hospitals. This showed that estimates of prevalence and seasonality of HMPV between and within given populations is highly variable and localised. As all the patients recruited in this study were admitted to PICU, the low incidence of HMPV

detection could reflect the reduced capability of HMPV to cause severe disease among young children. As most of the clinical diagnosis in this study was of severe pneumonia, this suggests that HMPV is an infrequent virus causing severe pneumonia in Sarawak. The observed HMPV detection rate was consistent with other studies that showed that HMPV was not a frequent agent of lung disease in children with or without underlying diseases, but the virus can elicit severe illness (Berkley *et al.*, 2010; Mathisen *et al.*, 2011; Wang *et al.*, 2014).

Some studies have found high co-detection rate between HMPV and RSV suggesting that it is a risk factor for severe disease (Greensill *et al.*, 2003; Semple *et al.*, 2007). The high incidence of HMPV/RSV co-detection is thought to be related to the partially overlap annual epidemics of both viruses, especially in temperate climates (Chan *et al.*, 2007; Mizuta *et al.*, 2013). However, William *et al.*, (2004) reported that single HMPV detection does not show appreciable differences with HMPV co-detection with other viruses and it did not aggravate the disease severity. Only one HMPV/RSV co-detection was found in this study. Furthermore, 45.1% of HMPV episodes were co-detected with an additional virus, with HMPV/HAdV co-detections the most frequently identified. Similar incidence of HMPV/HAdV co-detection was observed in children with RTI in Cambodia and Japan (Arnott *et al.*, 2013; Kaida *et al.*, 2007).

Pneumonia was the most frequent clinical diagnoses among all of the age groups studied. In a 25-year retrospective study of HMPV among young children in USA reported that HMPV was associated with pneumonia in 8% (Williams *et al.*, 2004). However, in a 2 years study in Thailand, among those 91% patients who required hospitalization, the primary manifestation of HMPV infection was also pneumonia. Similar with the study

done by Arnott *et al.*, (2013) and Wolf *et al.*, (2006), there is a strong association between HMPV infection and pneumonia.

In Malaysia, it was reported that the predominant subgroup is HMPV A2 (Chow *et al.*, 2016; Vinomarlini *et al.*, 2014). Predominant genotypes are replaced every 1-3 years within a given population (Arnott *et al.*, 2013; Nor'e *et al.*, 2014). The distribution of HMPV genotype was not identified in this study. Further study by analysing the G or F gene of HMPV is needed so as to evaluate the subgroup changes or to understand the predominant circulating HMPV genotype in Sarawak.

4.8 Human coronavirus (HCoV)

Three out of five known HCoV species were detected by RT-PCR assay in this study. The HCoV prevalence of 2.3% found in this study echoes previous studies which ranged from 2.1% to 8.4% where HCoV were identified as significant etiological agents of influenza-like-illness (ILI) and ARI (Chiu *et al.*, 2005; Gaunt *et al.*, 2010; Lau *et al.*, 2006; Mackay *et al.*, 2012; Sipulwa *et al.*, 2016; Suwannakarn *et al.*, 2014). Of the HCoV positive episodes, the majority were HCoV-OC43 (n=10, 0.86%), followed by HCoV-229E (n=9, 0.77%) and HCoV-NL63 (n=8, 0.69%). There was relatively equal prevalence of HCoV-OC43, HCoV-229E and HCoV-NL63. No HCoV-HKU1 was detected. Comparing current PCR data from other parts of the world, the prevalence of the different HCoVs appears to vary by year and location (Lau *et al.*, 2006; Matoba *et al.*, 2015; Sipulwa *et al.*, 2016). Nevertheless, HCoV-OC43 was the most frequent observed HCoV and this agrees with many previous studies which reported HCoV-OC43 as the predominant species (Gaunt *et al.*, 2010; Lau *et al.*, 2006; Prill *et al.*, 2012; Sipulwa *et al.*, 2016). This was

followed by HCoV-229E and HCoV-NL63 with similar incidences. This finding showed that HCoV-OC43, HCoV-229E and HCoV-NL63 contribute to a significant proportion of hospitalization due to respiratory illnesses in our locality.

There may be annual variations in the circulation of individual HCoV species. In Japan, HCoV-HKU1 displayed clear biennial peaks in January and February 2010 and in February and March 2012. The peak detection of HCoV-OC43was observed in November 2010, while that of HCoV-229E was in March 2013 (Matoba *et al.*, 2015). In temperate country, HCoV generally displayed marked winter seasonality between the months of December and April with exception of HCoV-229E which occur sporadically (Gaunt *et al.*, 2010). In Kenya, HCoV-OC43 was detected in most months in 2009–2011, but was not detected in 2012. While HCoV-NL63 predominated from February to June 2009, then disappeared and resurfaced in September 2012. In addition, HCoV-HKU1 was detected throughout the year and HCoV-229E detected with peaks in July and December (Sipulwa *et al.*, 2016). In Thailand, HCoV was detected throughout the 2-year study period from 2012-2013 and generally peaked from May to October, which coincided with the rainy season (Soonnarong *et al.*, 2016). The kind of coronaviruses that are the most frequent cause of infections changes in particular years. The study performed by Cabeça *et al.*, (2013) in Brazil in 2004-2008, revealed that the coronavirus responsible for infections in 62% of patients was mainly HCoV-229E while in 2008 it was HCoV-NL63 (71%). In our study, there were no obvious seasonal peaks associated with HCoV observed probably due to the small number of positive HCoV episodes. However, similar with the study done by Sipulwa *et al.* (2016), HCoV-NL63 predominated during the early season of the study in November to December 2012, disappeared and resurfaced after two years in June 2015. Additional multiyear studies on the prevalence of HCoV in the study hospitals are needed

to ascertain the seasonal and any possible inter-year variation in the relative incidences of the different coronaviruses.

In a study comparing HCoV among older children and adults in Ghana with upper respiratory tract infections, HCoV-229E and HCoV-OC43 showed higher detections rates among cases compared to control. These findings are similar with some studies in developed countries which have reported HCoV-OC43 and HCoV-229E as being commonly associated with RTI involving pneumonia which are consistent with our study as well (Owusu *et al.*, 2014; Suwannakarn *et al.*, 2014; van Elden *et al.*, 2004). Furthermore, HCoV-OC43 was detected in patients with a range of clinical conditions but predominated among those with chronic conditions and manifested as both URTI and LRTI (Mackay *et al.*, 2012).

On the other hand, HCoV-NL63 and HCoV-HKU1 were more prevalent in severe RTI of immuno-compromised individuals, elderly and infants. Based on numerous analysis on HCoV-NL63 infections by van der Hoek *et al.*, (2010), HCoV-NL63 occurs most frequently among children up to 3 years of age with co-existing diseases such as whooping cough, diabetes, myocarditis. Thus, this virus was more infectious among children with lowered immunity. Besides that, it was reported that HCoV-NL63 was most common among those with cough and is associated with croup (Chiu *et al.*, 2005; Van Der Hoek *et al.*, 2005). However, we did not find any cases of HCoV-NL63 in association with viral croup.

Another novel coronavirus, HCoV-HKU1, which resembled HCoV-229E in its structure, is the etiologic factor of severe pneumonia with acute respiratory failure. Frequent convulsions in the course of infection are the characteristic feature of the disease.

It frequently causes super-infections in children with bacterial bronchiolitis and asthma, which indicates infections mainly in people with lowered immunity. Besides that, it was reported that upper respiratory infection was the most common presentation of HCoV-HKU1 infections (Lau *et al.*, 2006; Lapinski, 2014).We reported no HCoV-HKU1 in this study partly because our study focused more on acute lower severe respiratory tract infections which required admission to PICU.

Similar to others, we observed a high number of co-detections (55.6%) with other respiratory viruses which involved mainly RSV. The high rate of co-detection with RSV here was previously demonstrated in other studies (Gaunt *et al.*, 2010; Kuypers *et al.*, 2007). They further explained that no differences in clinical outcome were observed for those HCoV/RSV co-detection compared with those singly infected with either virus, and so RSV presumably facilitates HCoV infections (or vice versa) without exacerbating morbidity. Among those HCoV species co-detection, it was observed that 70% of HCoV-OC43 were singly detected in most of the positive episodes in contrast to other HCoV species. This draws our attention as it might represent that HCoV-OC43 could play a crucial role in the pathogenesis of acute lower respiratory tract infections.

In conclusion, we demonstrate that HCoV contribute to hospitalization of children with ALRI and that the newly recognized HCoV-NL63 is at least as important as other HCoV species in this regard. HCoV-OC43 should be considered in the diagnosis of ALRI while HCoV-NL63 and HCoV-229E may play a role in more severe disease involving multiple viruses.

4.9 Human bocavirus (HBoV)

We detected HBoV in 26 (2.2%) of 1165 children with severe ALRI admitted to PICU in Sarawak. The proportion of respiratory specimens from symptomatic hospitalized children containing HBoV ranged from 1.5% to 19% (Bastien *et al.*, 2006; Hengst *et al.*, 2008; Kaplan *et al.*, 2006; Völz *et al.*, 2007; Zhang *et al.*, 2008). Sweden reported a prevalence of 3% among 24 of 806 pediatric (Allander *et al.*, 2005), while Australia 5.6% among 18 of 324 children < three years old (Sloots *et al.*, 2006). Kaplan *et al.* (2006) detected HBoV in 57 (18.3%) of 312 children with ARI severe enough to require hospital admission. In Peninsular Malaysia, HBoV was detected in 5.6% of 125 hospitalized children with acute respiratory disease in 2012 (Rogayah *et al.*, 2014). These data support an association between the virus and ALRI.

Most (92.3%, 24/26) children detected with HBoV were significantly younger than 24 months which is similar with other studies done (Kesebir *et al.*, 2006; Wang *et al.*, 2010).In this study, the patients who suffered from pneumonia comprised more than half (73.1%) of the positive HBoV episodes. This agrees with other studies which described the clinical symptoms of HBoV mostly related with wheezing, fever, bronchiolitis and pneumonia (Fry *et al.*, 2007; García-García *et al.*, 2012; Völz *et al.*, 2007).

The seasonal distribution of HBoV has not yet been clearly demonstrated. HBoV has been detected throughout the whole year, with variable peak season from year to year and from study to study (Lüsebrink *et al.*, 2009; Pozo *et al.*, 2007; Schildgen *et al.*, 2008). Some studies reported there was no obvious seasonal occurrence of HBoV (Bastien *et al.*, 2006; Zhang *et al.*, 2008). As reported by Rogayah *et al.*, (2014), HBoV was found to be circulating in Malaysia and the incidence appeared to occur during the dry season.

However, it is not feasible to draw conclusions concerning the epidemiology of this newly identified virus based on just a snapshot analysis of single season.

The results of our study reported a co-detection rate of 65.4%. Such high percentages have been reported by most studies with a co-detection rate of 18% to 90% (Allander *et al.*, 2005; Fry *et al.*, 2007; Schildgen *et al.*, 2008). Co-detection of HBoV with other common respiratory viruses was frequently detected among RSV, HAdV and HRV as seen in our study. Fry *et al.* (2007) observed 40 cases of co-detection with other viruses among 44 HBoV positive hospitalized children aged five years and below suffering from pneumonia. Moreno *et al.*, (2014) reported a co-detection rate of 46% among children under 2 years of age hospitalized due to an ARI during 2011. The high co-detection rate with other viruses could be attributed to seasonal co-circulation of those particular viruses. However, other authors also suggested that prolonged viral shedding could account for these findings (Blessing *et al.*, 2009; Martin *et al.*, 2010). Furthermore, together with the detection of the virus in asymptomatic individuals initially questioned the pathogenic role of HBoV (Chonmaitree *et al.*, 2008; Martin *et al.*, 2013). Nevertheless, a strong correlation between the respiratory disease and viral load ($>10^4$DNA copies/ml) has been shown as a significant predictor of LRTI (Jiang *et al.*, 2016). In their study, high virus load were noted solely in HBoV single detection suggesting the causative role for HBoV whereas low virus load were more detected in virus co-detection. Furthermore, HBoV has been detected as the third etiologic agent causing confirmed pneumonia (Esposito *et al.*, 2013; García-García *et al.*, 2012) and thus, all of them support the pathogenic role of this virus. Further study on viral load will give an insight into the causal role of HBoV in association with the high co-detection rate found in this study.

4.10 Influenza virus (IFV)

In this study, IFV detection accounted for only 1.9% of ALRI among the hospitalized episodes. The overall burden of IFV to ALRI in other studies was in accordance with our study. In a 3-year prospective study of children younger than 24 months hospitalized with febrile respiratory illness found only 1.5% (3 out of 201 children) with influenza infection (Suryadevara *et al.*, 2011). Another meta-analysis of 9 studies in which IFV was lab-confirmed, the burden of IFV was estimated to be 3% among those hospitalized ALRI in children (Lukšić *et al.*, 2013). In a prospective study conducted among 51 children < 3 years old with ALRI using real time PCR, only 2 cases (4.5%) of IFV was found (Meligy *et al.*, 2016). However, similar with other respiratory viruses, IFV was most frequently detected among age group of 1-12 months due to their immature immunity system.

Although certain studies reported high prevalence of IFV, these studies frequently present a systematic age pattern which suggested that RSV is more important in infancy, with a gradual shift to IFV by about 5 years of age as the pathogen more likely to cause severe respiratory illness (Simonsen *et al.*, 2011). Furthermore, in another study, RSV, HMPV and HPIV were more commonly isolated from children less than 4 years old, while IFV was isolated in 7.3-8.2% among those school-aged children (Mizuta *et al.*, 2013). In Bangkok, Prachayangprecha *et al.*, (2013) also reported the prevalence of IFV was predominant in the school-age group. In Peru and Africa, studies which involved influenza-like-illness (ILI) across all age group reported that the detection of IFV was significantly higher among patients older than 5 years old (Laguna-Torres *et al.*, 2009; Njouom *et al.*, 2012). This explained the low prevalence observed in this study which focused mainly on patients younger than 5 years old with severe ALRI admitted to PICU.

In addition, IFV were more commonly detected in URTI and among those with mild or moderate respiratory symptoms (Lu *et al.*, 2013). In Malaysia, Rahman *et al.*, (2013) revealed that out of 65 IFV positive results, patients of mild respiratory symptoms accounted for 32.3% and 53.58% with moderate symptoms while only 16.94% were of severe respiratory symptoms. In another study of 132 children admitted with influenza in Malaysia, 16 (12.1%) had severe disease requiring intensive care or ventilation (Sam *et al.*, 2010).

Recent data from tropical countries demonstrated that the seasonality of IFV corresponds mostly with the rainy season and only a few cases detected during the dry season (Dosseh *et al.*, 2000; Mardy *et al.*, 2009; Njouom *et al.*, 2012). In Peninsular Malaysia, there is year-round influenza activity with no clear seasonal trends (Saha *et al.*, 2014; Sam, 2015). This is consistent with our study although the IFV prevalence was low. Influenza A is usually detected more frequently than influenza B, although year-to-year variation may be considerable. We observed a similar rate of both IAV and IBV in this study. It was shown that SGH detected only IBV throughout the study periods, whereas the other two hospitals shown equivalent distribution of IAV and IBV. Sam *et al.*, (2015) in a study examining the evolution of IBV in Malaysia demonstrated that the incidence of influenza B in their study group was generally lower than influenza A infection; however, higher incidence of influenza B was observed in 2000, 2004, 2005 and 2007 coinciding with shifts in the predominant lineage. Only one case of influenza C was observed in this study. This might be due to the reason that IVC usually causes inflammation of the upper respiratory tract, and only occasionally the virus spreads to the lower respiratory tract and causes bronchitis and bronchopneumonia (Gouarin *et al.*, 2008; Kauppila *et al.*, 2014; Matsuzaki *et al.*, 2007).

In summary, although influenza virus was not the main contributor for children younger than 5 years and below with ALRI, it's still capable of causing lethal disease (CDC, 2019). Continuous population-based surveillance of influenza viruses is needed to properly define the temporal pattern of influenza virus circulation and further study on the phylogenetic and antigenic studies of influenza viruses can increase our understanding of the emergence and spread of new influenza drift variants locally. Also we need to take into account the cost effectiveness of some vaccine preventable viral illnesses in our developing country, to redirect our financial resources to the most prevalent viruses in the region with appropriate risk group.

In this study, there were 22.6% of negative episodes being documented which could possibly be attributed to bacteria, atypical microorganisms or other virus infections other than the 14 respiratory viruses investigated in the present study. Previous studies on ALRI have demonstrated bacterial detection rates between 6% and 26% in these cases (Boldy et al., 1990; Creer et al., 2006; Macfarlane et al., 2001). These bacterial detection rates are in agreement with the percentage (22.6%) of negative episodes observed in the present study.

4.11 Viral co-detection

The recent introduction of more sensitive molecular methods in the clinical practice has increased the virus detection rates significantly compared to viral culture methods. This has, however, made the interpretation of positive test results more difficult, especially when multiple viruses are found. Viral co-detection was seen in 270 (30%) of the 902 positive episodes in our study. As mentioned earlier in section 4.1, the clinical significance

of such viral co-detection and possible co-detection is unclear, and review of the literature is conflicting. Certain viruses were shown clinically significant when co-detected for example RSV and HAdV whereas some were more questionable as they present at high rates in asymptomatic patients as reviewed in literature for example HRV and HBoV (Aberle *et al.,* 2005; Nascimento *et al*., 2010; Ruuskanen *et al*., 2013; Martin *et al*., 2013).

Even though the impact of viral co-detection on severity of disease is unclear, the need for rapid and accurate detection of a wide spectrum of viral respiratory pathogens is desirable to understand their correlation with clinical illness and for epidemiologic and hospital infection control reasons. The field of clinical virology has changed dramatically since introduction of new diagnostic techniques in the clinical laboratory. Newer assays are still being developed to expand the number of detected targets to include viral and bacterial respiratory pathogens such as HPIV-4, coronavirus, HPMV and HBoV with decreased turnaround time. This will further increase our understanding of the circulating respiratory pathogens during different seasons of the year and in different health care settings (hospital based vs community based) and potentially help in the development of new vaccines. Continued advances in diagnostic technology have enabled diagnostic microbiology laboratories to expand their capability of detecting respiratory viruses and thus help understand the epidemiology of respiratory viruses. It is evident that many unanswered questions still need to be addressed: for example, what is the scientific reason behind the seasonality of the different respiratory viruses, and which antiviral treatments should be used, if any, to treat these viral respiratory tract infections? Despite progress made in the 20th and 21st centuries, there is still no common oral antiviral therapy available for the treatment of these respiratory viruses except for influenza. Further studies need to be conducted to answer these questions. The study was limited by the fact that no clinical data

were associated with test results; thus, we could not assess symptoms associated with viral infection or co-detection disease progression, treatment plans, and response to treatment.

4.12 Concluding remarks

Pediatric ALRI represents the main causative mortality in children under five years worldwide (Troeger *et al.*, 2017). This highlights ALRI as an important area for research and has been chosen as a primary focus for WHO to implement a research agenda in order to succeed in the battle against respiratory viruses (WHO 2013). In this study, we described the epidemiology of viral pathogens of ALRI as well as characterising the clinical and demographic features of respiratory viruses. We also sought to examine the molecular genetics characterization of main respiratory viruses detected in Sarawak compared to those identified elsewhere. By adding to the body of knowledge it will enable further work to be carried out into the causative agents of ALRI and the association between clinical symptoms and respiratory viruses. Full assessment of the causative viral agents of ALRI is important in targeting treatments in ALRI. Comparatively less is known about viral agents in ALRI. The development of PCR as a diagnostic technique has allowed high throughput rapid viral diagnostics in virology centres worldwide. We collected NPAs, NS or either ETT from children present with ALRI who had been admitted to PICU ward between November 2012 and October 2015. In this setting viral diagnostics are not usually available. However, samples were transported to IHCM, UNIMAS where multiplex PCR was done for detection of respiratory viruses.

We were successful in analysing those viral agents and demonstrated high detection rates in 77.4% of the 1165 hospital admission episodes. The most common viruses

detected were RSV, HAdV and HRV. We also reported high levels of co-detection with 30% of children with positive viral detection having more than one virus detected in PCR. Our findings highlight the wide variety of viruses that are detected in children presenting with ALRI. These findings also raised questions to the role of co-detection in our study and highlight the need for further assessment of co-detection in pediatric ALRI.

Further analysis was successfully achieved in characterizing the molecular genetics of RSV, HAdV and HRV. Results from this current study show that RSV A predominated over RSV B over the study period. It has become apparent that it is important to understand the epidemiology of RSV caused by different subtypes and strains of viruses causing ALRI because more therapeutic and prophylactic modalities against viruses like RSV have become available (Higgins *et al.*, 2016; Turner *et al.*, 2014). For HAdV, our study indicates species B was the most frequent HAdV circulating in Sarawak. These results support the need for continued surveillance and development of vaccines with broad protective immunity. The health sector should consider introducing adenovirus vaccine containing HAdV types 3 and 7 components to be administered to pediatrics in Sarawak since they are the most vulnerable in case of an outbreak. A total of 77 HRV genotypes (38 HRV-A, 2 HRV-B and 37 HRV-C) were identified. HRV-A and HRV-C were predominantly found in our study with both displaying a high genetic variation in Sarawak. The presence of many diverse strains of HRV has become apparent, and it is likely that more will emerge. Genotypic assignment and identification of HRV types will facilitate monitoring of emerging novel variants, and investigations into type-associated differences in epidemiology, transmission and outcomes.

CHAPTER 5

CONCLUSION, LIMITATIONS AND RECOMMENDATIONS

5.1 Conclusion

In conclusion, this three years study provided an important basic profile of the epidemiology, seasonality, age distribution, co-detection patterns as well as clinical association of viral respiratory infections in hospitalized children with ARTI in Borneo, Sarawak. The present study described the contribution of respiratory viruses to the burden of severe ALRI within the pediatric population in Sarawak, which accounted for 77.4% of respiratory infections during the 3-year study period. Among those positive episodes, RSV, HAdV, HRV and HPIV accounted for 91.5% of paediatric viral ALRIs among children below five years old who were admitted to PICU ward despite the low prevalence of influenza virus. These viruses also contribute to the economic burden of healthcare. Therefore, just like influenza, priority should be focused on RSV, HAdV and HRV as well.

The prevalence of the viral distribution varied across different age groups studied. Although high prevalence of respiratory viruses were observed among those less than 2 years old, children under 1 year of age in this study are more vulnerable to RSV and HRV infections according to age group.

There were overlapping seasonal trends observed between RSV and HAdV throughout the study period. HAdV and HPIV were positively associated with temperature and detected mainly during the hot months. These observations are intriguing and demonstrate that the implementation of a children based ALRI surveillance system or a subsequent longitudinal study in the future could assist to observed time-dependent

circulation of respiratory viruses. These data can be useful to initiate precaution measures (hygiene, exposure prophylaxis) to prevent spread of infections in high risk groups.

This study provides additional molecular characterization data for RSV, HAdV, and HRV/EV. RSV strains co-circulated during the 3-year study period but their numbers fluctuated from season to season with RSV-B predominant during the first season and RSV-A predominant in the next two seasons. Besides that, we found HAdV species B predominated over HAdV species C in this study. HAdV-7 was the most predominant in 2012-2013 season and HAdV-3 was more prevalent in 2015 during the two peak periods. HRV displayed a broad genetic diversity with many strains circulating in a single year only. Phylogenetic analysis of HRV showed that several different genotypes of HRV were detected including outbreak-like clusters such as HRV-A12 and HRV-Cpat_10. There were several HRV-A which were not so closely associated with the known genotypes. However, in the absence of the VP1 sequence, the putative new types are considered provisionally assigned. As expected, a high number of HRV types including seven new provisionally assigned types were identified which will be further sequenced in a separate study at the institute to confirm type assignment. HRV mainly belonged to species A and C in this study. Most of the EV68 were clustered phylogenetically within clade B which were more likely to be detected in the typical enterovirus season mainly in 2013-2014 while clade A viruses were nearly seen throughout the year. In general, viruses are continuously evolving and circulating all over the world, in most instances the origin of new emerging variants is unclear. Molecular data from this study may provide insights into viral evolution in future study and they may help for vaccine development, especially needed in developing countries. To investigate the persistence, displacement or new emerging virus strains, consecutive or virus-related long-term molecular studies are needed.

This study investigated the role of eight respiratory viruses including those rarely identified viruses such as HMPV, HRV-C, HBoV and HCoV among those with ALRI using multiplex RT-PCR approach. The high co-detection rate (30%) of respiratory viruses has led to the speculation that the presence of multiple viruses in one respiratory specimen may affect the clinical presentation of ALRI. Viruses might interact indirectly or directly, and the effect may depend upon which viruses co-infect. The detection of multiple viruses highlights the need for prospective surveillance and routine diagnostic to help the physician in making appropriate diagnosis in relation to the main virus responsible for the disease.

In this study, pneumonia remains a leading cause of ALRIs in Sarawak mainly caused by RSV and HAdV. Acute bronchiolitis was mainly associated with RSV while HAdV, HRV and HPIV was associated with acute exacerbation of bronchial asthma, newly diagnosed bronchial asthma and viral wheeze to a similar degree. It was also found that croup was predominantly associated with HPIV-1. The ability to ultimately identify the viral etiology of ALRI and differentiate between these infections by clinical manifestations is fundamental to effective treatment if available. Accurate and rapid diagnosis of these viruses can help physicians optimize patient treatment and outcomes. From a public health perspective, information on the prevalence and seasonality of respiratory viruses are crucial to be used as a basis to optimize the timing of interventions for future vaccination programs such as RSV vaccines.

5.2 Limitations and shortcomings

There are several limitations to our study. Firstly, bacterial pathogens were not included in our study which might underestimate the association of ALRI with mixed viral-bacterial infections. Secondly, healthy or asymptomatic controls were not included in the study. As certain viruses were found also in asymptomatic patients thus their viral carriage burdens and the main causative role of virus as pathogen could not be elucidated. Thirdly, owing to ethical considerations, invasive procedures to obtain samples directly from lower respiratory tract were not performed. The detection of viruses in nasopharyngeal aspirates or nasal swab by PCR assay could represent upper respiratory tract or convalescent-phase shedding, and thus detection may not denote causation. Fourthly, packaging, handling and transport of the viral specimens from the three hospitals might have contributed to variation in the positive rate of respiratory viruses identified. Lastly, we have not yet collected and analysed all the clinical information available in the database of this study. Certain information on the disease outcomes (eg. the death records) were not included and thus may underestimate the severity and disease burden.

5.3 Recommendations for future studies

Future studies may include quantification of the viral load to elucidate the role of each virus especially in co-detection and severity of illness. Real-time PCR should be considered to estimate viral loading, thereby allowing a quantitative comparison for the real causative agent of illness in viral co-detection and comparison of disease severity. Besides, bacterial-viral co-detection and mycoplasma-viral co-detection should be considered as well to estimate the etiology and interactions involved in ARTIs. Data analysis of clinical manifestations and laboratory results should be further analysed in association to the causative agent of respiratory viruses and co-detection. Further characterization of RSV genotypes can provide a better understanding of the molecular epidemiology of RSV. Finally, it is necessary to highlight the importance of long-term surveillance of respiratory viruses especially for RSV and HAdV in Borneo, Sarawak and also having baseline data to monitor trends for future intervention such as vaccination program.

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APPENDICES

Appendix A

JAWATANKUASA ETIKA & PENYELIDIKAN PERUBATAN
(Medical Research & Ethics Committee)
KEMENTERIAN KESIHATAN MALAYSIA
d/a Institut Pengurusan Kesihatan
Jalan Rumah Sakit, Bangsar
59000 Kuala Lumpur

Tel. : 03 2282 9082/03 2282 9085
03 2287 4032/03 2282 0491
Faks : 03 2287 4030

Ruj. Kami : (7) dlm.KKM/NIHSEC/08/0804/P13-369
Tarikh : 10 September 2013

Prof Madya David Perera
Institut Kesihatan dan Perubatan Komuniti
Universiti Malaysia Sarawak (UNIMAS)

Tuan,

NMRR-12-1298-13465
Protocol No: IHMCM-RESP-2013-001
A PROSPECTIVE STUDY ON THE EPIDEMIOLOGY OF SEVERE COMMUNITY-ACQUIRED VIRAL LOWER RESPIRATORY TRACT INFECTION IN CHILDREN AGED 5 YEARS AND BELOW IN SARAWAK

Dengan hormatnya perkara di atas adalah dirujuk.

2. Bersama dengan surat ini dilampirkan surat kelulusan saintifik dan etika bagi projek ini. Segala rekod dan data subjek adalah SULIT dan hanya digunakan untuk tujuan kajian dan semua isu serta prosedur mengenai *data confidentiality* mesti dipatuhi. Kebenaran daripada Pengarah hospital di mana kajian akan dijalankan mesti diperolehi terlebih dahulu sebelum kajian dijalankan. Puan perlu akur dan mematuhi keputusan tersebut.

3. Penyelidik-penyelidik bersama bagi projek ini adalah seperti berikut:
- Dr Ooi Mong How
- Dr Wong See Chang
- Dr Anand Mohan
- Chai May Yin

4. Adalah dimaklumkan bahawa kelulusan ini adalah sah sehingga 10 September 2014 dan Tuan perlu menghantar *'Continuing Review Form'* setiap tahun bagi memperbaharui kelulusan etika. Pihak Tuan juga perlu mengemukakan laporan tahunan, laporan tamat kajian dan juga laporan mengenai "*All adverse events, both serious and unexpected*" kepada Jawatankuasa Etika & Penyelidikan Perubatan, KKM.

Sekian terima kasih.

BERKHIDMAT UNTUK NEGARA

Saya yang menurut perintah,

(DATO' DR CHANG KIAN MENG)
Pengerusi
Jawatankuasa Etika & Penyelidikan Perubatan
Kementerian Kesihatan Malaysia

JAWATANKUASA ETIKA & PENYELIDIKAN PERUBATAN
(Medical Research & Ethics Committee)
KEMENTERIAN KESIHATAN MALAYSIA
d/a Institut Pengurusan Kesihatan
Jalan Rumah Sakit, Bangsar
59000 Kuala Lumpur

Tel. : 03 2282 9082/03 2282 9085
03 2287 4032/03 2282 0491
Faks : 03 2287 4030

Ref: (8) dlm.KKM/NIHSEC/800-2/2/2 Jld 2.P13-369
Date : 10th September 2013

NMRR-12-1298-13465
Protocol No: IHMCM-RESP-2013-001
A PROSPECTIVE STUDY ON THE EPIDEMIOLOGY OF SEVERE COMMUNITY-ACQUIRED VIRAL LOWER RESPIRATORY TRACT INFECTION IN CHILDREN AGED 5 YEARS AND BELOW IN SARAWAK

Principal Investigator: Associate Prof Dr David Perera
Institute of Health and Community Medicine
Universiti Malaysia Sarawak (UNIMAS)

Documents received and reviewed with reference to the above study:

1. Protocol Amendment version 3 dated 17th July 2013.
2. Patient information sheet (English) & Informed Consent Form (English) version 3 dated 17th July 2013.
3. Patient information sheet (BM) & Informed Consent Form (BM) version 3 dated 17th July 2013.
4. Study Clinical Report Form version 1 dated 12th March 2013.
5. Curriculum vitae and IA-HOD-IA form of all investigators.

The Medical Research & Ethics Committee, Ministry of Health Malaysia operates in accordance to the International Conference of Harmonization Good Clinical Practice Guidelines.

Project Sites: Sarawak General Hospital, Sibu Hospital, Bintulu Hospital

Comment: Please note that the approval is valid until 10th September 2014. To renew the approval, a completed 'Continuing Review Form' (Appendix 1) has to be submitted to MREC at least 2 months before the expiry of the approval.

Decision by Medical Research & Ethics Committee:
(√) Approved (Exempted from Full Board Review)
( ) Disapproved

Date of Decision: 10th September 2014

(DATO' DR CHANG KIAN MENG)
Chairman
Medical Research & Ethics Committee
Ministry of Health

MEMBERS OF THE MEDICAL RESEARCH & ETHICS COMMITTEE WHO REVIEWED THE DOCUMENTS / PROTOCOL

NAME	DESIGNATION	GENDER
Dr Afendi Dahlan	Lecturer, Faculty of Pharmacy, Cyberjaya University College of Medical Sciences	Male
Dr Tang Swee ping	Consultant Paediatrician & Paediatric Rheumatologist, Dept of Paediatrics,Hospital Selayang	Female
Mdm Yam Phak Hong	Laymember	Female

The MREC is constituted in compliance with ICH GCP and local regulatory requirements. Any member of the MREC who is involved in the study / project under review will not participate in the approval documents related to the study / project

Appendix B

CONSENT TO PARTICIPATE IN A RESEARCH STUDY

Study Title: A Prospective study on the Epidemiology of Severe Community-Acquired Viral Lower Respiratory Tract Infection in children Aged 5 years and below in Sarawak.

This research study involves three study sites namely SGH, Sibu and Bintulu. Your study doctor, Dr Ooi Mong How and Dr Chor Yek Kee (SGH), Dr Wong See Chang (Sibu Hospital) and Dr Anand Mohan (Bintulu Hospital) will explain this study to you.

Medical research studies include only people who choose to take part. Take your time to make your decision about participating. You may discuss your decision with your family and friends and with your health care team. If you have any questions, you may ask your study doctor.

Your child is being asked to take part in this study because he/she might have acute lower respiratory tract infection.

Why is this study being done?

This study is conducted to help understand the different respiratory viruses that account for acute lower respiratory tract infection in children. We would like to study the clinical and molecular epidemiology of respiratory viral infections in young children aged 5 years and younger in Sarawak besides developing better molecular diagnostic assays for rapid identification of these viruses.

What will happen if my child takes part in this research study?

If you agree to let your child take part in this study, you will first sign this Informed Consent Form before any study-related procedures are performed. Nasopharyngeal aspirates and swab or endotracheal tube (ETT) secretion (if your child is on mechanical ventilation) will be drawn from your child for cell culture and virological assays to help with the characterization of the respiratory virus that responsible for the disease. Apart from the data collection, there will be no further requirements from you. Unused or leftover samples will be stored for possible future use in the study team's own research.

How long will my child be in the study?

Participation in the study will take about 5 minutes to collect nasopharyngeal aspirates and swab or endotracheal tube samples (if any) from your child.

Can my child stop being in the study?

Yes. You can decide on behalf of your child to stop at any time. Tell the study doctor if you are thinking about stopping or decide to stop.

What side effects or risks can I expect for my child being in the study?

There is minimal risk to your child as the study involves basic sample collections which is part of the routine for normal patient care procedure. Sample collection may cause temporary discomfort from the catheter, and very rarely infection. There is a risk of loss of privacy based on data collection. However, the information about the child will be handled as confidentially as possible. Your child's name will not be used in any published reports or shared with any outside institution.

Are there benefits to taking part in the study?

There is no direct benefit to you. However, information gained from this study will help improve the scientific knowledge about acute respiratory viral infection in children.

What other choices do my child have if my child do not take part in this study?

As an alternative, you can choose not to participate in this study.

Will my child's medical information be kept private?

We will do our best to make sure that the personal information in medical record is kept private. However, we cannot guarantee total privacy. Your child's personal information may be given out if required by law. If information from this study is published or presented at scientific meetings, your child's name and other personal information will not be used. Information from this study will be submitted to the Institutional Review Board (IRB). Medical records and all other relevant study materials which identify your child, including this Consent Form signed by you, might be inspected by any entity legally entitled to review them. Because of the need to release information to these parties, absolute confidentiality cannot be guaranteed. The results of this research project may be presented at meetings or in publications; however, your child's identity will never be disclosed in such presentations.

What are the costs of taking part in this study?

Your child will not be charged for any of the virological investigation related to this study.

Will my child be paid for taking part in this study?

Your child will not be paid for taking part in this study.

What are my child's rights if he/she takes part in this study?

Taking part in this study is your child's choice. You may choose on behalf of your child either to take part or not to take part in the study. If you decide on behalf of your child to take part in this study, you may leave the study at any time. No matter what decision you make, there will be no penalty to you and you will not lose any of your regular benefits. Leaving the study will not affect your child's medical care. Your child can still get his/her medical care from our institution.

CONSENT

You have been given a copy of this consent form to keep. PARTICIPATION IN RESEARCH IS VOLUNTARY. You have the right to decline to participate or to withdraw at any point in this study without penalty or loss of benefits to which you are otherwise entitled.

If you wish to participate in this study, you should sign below.

_____ _____

Date Participant's Signature for Consent

_____ _____

Date Person Obtaining Consent

I have read the information, or it has been read to me. I have had the opportunity to ask questions about it and my questions have been answered to my satisfaction. I consent voluntarily to have my child's samples stored in the manner and for the purpose indicated above.

Print Name of Participant _____

Signature of Participant _____

Date _____

Day/month/year

If illiterate

A literate witness must sign (if possible, this person should be selected by the participant and should have no connection to the research team). Participants who are illiterate should include their thumb-print as well.

I have witnessed the accurate reading of the consent form to the potential participant, and the individual has had the opportunity to ask questions. I confirm that the individual has given consent freely.

Print name of witness_____ AND Thumb print of participant

Signature of witness _____

Date _____

Day/month/year

Universiti Malaysia Sarawak
Institut Kesihatan dan Perubatan Komuniti
Jalan Dato Mohd Musa, Kota Samarahan, Sarawak
Tel: 082-582351, Fax: 082-671 785
Contact persons: a. Assoc Prof Dr David Perera (Director, email: davidperera@yahoo.com)
b. Chai May Yin (Researcher, email: cmy_1010@yahoo.com)

Patient Biodata

Name:

Address:

Gender: Male / Female

Date of Birth: Age:

Mother's IC No /Adult IC No: Hospital Registration No:

Ethnic Group: Iban/Chinese/Malay/Melanau/Bidayuh/Kayan/Kenyah/Penan/Indonesian/Others: _____

Clinical Syndrome (Please Tick) Date of onset (earliest symptom):_____

[ ] Pneumonia

[ ] Acute Bronchiolitis

[ ] Viral Croup

[ ] Recurrent Viral Wheeze

[ ] Newly Diagnosed Bronchial Asthma with Concurrent Respiratory Tract Infection

[ ] Acute Exacerbation of Bronchial Asthma Secondary to Respiratory Tract infection

[ ] Others, Please Specify:

Sample Types (Please Tick)

Date of Collection: _____

[ ] Nasopharyngeal Swab

[ ] Nasopharyngeal Aspirate

[ ] Endotracheal Tube Secretion

[ ] Others, Please Specify: _____

Requesting Doctor's Information

Hospital: SGH / Sibu / Bintulu / Miri / Others: _____

Name of The Doctor: _____

Name of The Specialist In Charge: _____

Patient's sticker

Appendix D Study No: _____/_____/_____

A Prospective Study on the Epidemiology of Severe Community-Acquired Lower Respiratory Tract Infection in Children Aged 5 years and Younger in Sarawak

DEMOGRAPHIC INFORMATION AT ENROLMENT		
Date of enrolment: ____/_____/_____ *day/month/year*		
Patient's Name:	**Age:**	**Date of Birth:**
	Gender: Male / Female	
Mother's Name:	**Race**: please [√]	
	Chinese [ ] Kenyah [ ]	
Mother's IC No.	Bidahyuh [ ] Kayan [ ]	
	Malay [ ] Penan [ ]	
Hospital Registration No.	Melanau [ ] Indonesian [ ]	
	Iban [ ] Others _____	
Place of Regular Stay		
Town [ ] Rural [ ] Plantation [ ]		
Tel. No.	**(Please specify any specific person to contact)**	

PRESENTING HISTORY (At any time of the illness)

Symptoms

[ ] Fever	[ ] Unable to drink / breast feed normally
[ ] Cough	[ ] Diarrhea
[ ] Sneezing	[ ] Abdomen distention
[ ] Running Nose	[ ] Concentrated urine
[ ] Fast breathing	[ ] Conjunctivitis / red eyes
[ ] Breathlessness / dyspnoea	[ ] Eye discharge
[ ] Noisy breathing	[ ] Ear discharge
[ ] Hoarseness of voice	[ ] Not as active as usual self/ lethargy
[ ] Wheezing	[ ] Seizures
[ ] Stridor	[ ] Abnormally sleepy / drowsiness
[ ] Sore throat	[ ] Inconsolable cry / irritability
[ ] Vomitting	[ ] Skin rashes

CONTACT HISTORY

Did the patient have recent contact with any of the following?

[ ] Anyone with recent respiratory illness

[ ] Anyone with recent febrile illness

[ ] Go to nursery/daycare/kindergartens

[ ] History of recent travel (within the last 2 weeks before the onset of illness)

If yes, where:_____

IMMUNIZATION

[ ] Completed for the age?

[ ] Influenza vaccine?

[ ] Pneumococcal vaccine?

PAST MEDICAL / FAMILY HISTORY

a) Birth history [ ] Term [ ] Prematurity

b) Birth weight [ ] < 2.5kg [ ] > 2.5kg

c) Past history of i) Wheeze [ ] Yes [ ] No

ii) Chest infection [ ] Yes [ ] No

d) Family history of asthma or atopy illness [ ] Yes [ ] No

e) Co-existing illness

[ ] Chronic lung disease [ ] Celebral Palsy

[ ] Prematurity [ ] Asthma

[ ] Congenital Lung anomalies, indicate pathology: _____ [ ] Congenital Heart Disease, indicate lesion:_____

[ ] TB

f) Passive smoking [ ] Yes [ ] No

NUTRITIONAL HISTORY (for those < 1 year)

a) Ever breasted [ ] Yes [ ] No

b) Duration of breastfeeding [ ] > 6 months [ ] < 6 months

VITAL SIGNS / ANTHROPOMETRY

a) Axillary temperature: _____ ˚C

b) Respiratory rate: _____ / min

c) Heart rate: _____ / min

d) SpO_2 at room air: _____%

e) SpO_2 at _____L/min O_2 **OR**

SpO_2 on ventimask _____%O_2 is _____%

PHYSICAL EXAMINATION

a) Conscious state:

[ ] Alert [ ] Response to verbal

[ ] Unconcious [ ] Response to pain

b) Height _____cm c) Weight _____kg

d) Sign	**Yes**	**No**	**Sign**	**Yes**	**No**
i. Dehydration			xi. Barking cough		
ii. Poor perfusion			xii. Chest wall in-drawing		
iii. Conjunctivitis			xiii. Intercoastal recession		
iv. Skin rashes			xiv. Ausculatation of lungs		
v. Infected throat			a) Reduced breath sound		
vi. Nasal flare			b) Crepitations		
vii. Audible wheeze			c) Rhonchi		
viii. Audible stridor			d) Stridor		
ix. Hoarseness of voice			e) Transmitted sound		
x. ETT secretions [ ] Dry [ ] Whitish [ ] Blood [ ] Yellowish			xv. Heart sound [ ] Normal [ ] Murmur		
			xvi. Hepatomegaly If yes: _____cm below costal margin		
			xvii. Splenomegaly If yes: _____cm below costal margin		

SUMMARY SHEET Study No: _____/_____/_____

NAME	
DATE OF ADMISSION	
DATE OF DISCHARGE	
DATE OF DEATH	

Number of days on oxygen therapy
(including days on positive pressure ventilation): _____

Outcome	[ ] Survived [ ] Death

Discharge Diagnosis (Please tick the relevant box)

[ ] Pneumonia

[ ] Bronchiolitis

[ ] Croup

[ ] Bronchitis

[ ] Recurrent viral wheeze

[ ] Newly diagnosed asthma with concurrent respiratory tract infection (RTI), Specify the diagnosis of RTI: (e.g. Pharyngitis, pneumonia)_____

[ ] Acute exacerbation of asthma secondary to RTI, specify the diagnosis of RTI (e.g. Pharyngitis, pneumonia): _____

[ ] Other, please specify _____

Investigation Results on Enrolment into the study

a) *Chest X-ray abnormalities*

[ ] Consolidation [ ] Air-bronchogram

[ ] Hyperinflation [ ] Atelactasis

[ ] Infiltrates [ ] Effusion

b) *Hemoglobin* _____g/dL

c) *TWC* _____x10^3/µl

Neut ____% Lymp ____% Eosino ____% Mono____%

d) *Platelets* _____ x10^3/µl

e) *Sodium* _____mmol/L

f) *Potassium* _____mmol/L

g) Urea _____mmol/L

h) *AST* _____U/L

i) *ALT* _____U/L

j) *Albumin* _____g/L

k) *Calcium* _____mmol/L

l) *CRP* _____mg/L

m) *Blood C& S*	
n) *ETT C& S*	

o) *ABG (on enrolment into the study)*

pH _____ HCO_3 _____

pCO_2 _____ BE _____

PO_2 _____

Investigation Results on Enrolment into the study (cont.)	
p) *RBS* _____mmol/L	
q) *Mycoplasma serology* On admission to PIC : _____ On discharge from PICU: _____ On discharge from ward : _____	
r) *ETT secretion*	
Date sent to UNIMAS	
Results:	
Other Investigations	