Molecular Epidemiology of Respiratory Viruses associated with Acute Lower Respiratory Tract Infections in Children from Ghana

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By

Evangeline Obodai

From Ghana

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1st Reviewer: PD. Dr. Thorsten Wolff

Department of Infectious Diseases

Unit17, Influenza and other Respiratory Viruses

Robert Koch-Institut

Seestrasse 10

13353 Berlin, Germany

2nd Reviewer: Prof. Dr. Rupert Mutzel

Department of Biology, Chemistry and Pharmacy

Institute for Biology - Microbiology

Freie Universität Berlin

Königin-Luise-Straße 12-16

14195 Berlin, Germany

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To the Glory of God

Dedicated to

My beloved family,

And

Motherland Ghana

Abbreviations

Virus	
FCV	Feline calicivirus
HAdV	Human adenovirus
HBoV	Human bocavirus
HCoV	Human coronavirus
HIV	Human immunodeficiency virus
HMPV	Human metapneumovirus
HPIV	Human parainfluenza virus
IV	Influenza virus
RSV	Respiratory syncytial virus
RV	Human Rhinovirus

Viral Genes	
CP	Capsid protein
DNA	Deoxyribonucleic acid
DPol	DNA polymerase
F glycoprotein	The fusion glycoprotein
G glycoprotein	The attachment glycoprotein
HA	Hemagglutinin
HN	Hemagglutinin- neuraminidase
L	Large polymerase
M	Matrix Protein
N	Nucleoprotein
NA	Neuraminidase
NEP	Non-structural export protein
NS	Non-structural protein
NP	Non-structural protein
ORF	Open reading frame
PAT	Provisionally assigned type
PA	Polymerase protein
PB1	Polymerase protein
PB2	Polymerase protein
P-Pol	Polyprotein
RNA	Ribonucleic acid
RNP	Ribonucleoprotein complex
SH	Small hydrophobic glycoprotein
UTR	Untranslated region
VP	Viral protein

Others

%	Percentage
µl	Microliter
µM	Micromolar
ALRI	Acute lower respiratory tract infection
ARD	Acute respiratory disease
CAP	Community acquired pneumonia
cDNA	Complementary DNA
CFR	Case fatality ratio
CPE	Cytopathic effect
dNTPs	Deoxynucleoside triphosphates
dNUTPs	dNTP with dUTP (deoxyuridine triphosphates)
EDTA	Ethylenediaminetetraacetic acid
FMCA	Fluorescence melting curve analysis
HA	Hemagglutination assay
HAU	Hemagglutination unit
HI	The hemagglutination inhibition
ILI	Influenza-like illness
kb	Kilo base
KBTH	Korle Bu Teaching Hospital
LRTI	Lower respiratory tract infection
MDCK-SIAT	Madin-Darby canine kidney-cDNA of human 2,6-sialtransferase
MEM	Minimum essential medium
ml	Milliliter
nM	Nanomoles
NPA	Nasopharyngeal aspirate
NS	Nasal swab
nt	Nucleotide
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PMLCH	Princess Marie Louise Children's Hospital
RBCs	Red blood cells
RTI	Respiratory tract infection
SARI	Severe acute respiratory illness
UK	United Kingdom
USA	Unites States of America
VR2	Second variable region
WHO	World health organization

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1 Introduction

1.1 Burden of acute lower respiratory tract infection in children

Acute lower respiratory tract infections (ALRI) are an important cause of illness and death in children worldwide [1, 2]. In 2010 episodes of ALRI have been reported to cause about 4.9 million hospital admissions in young children worldwide [1]. There were approximately 1.4 million deaths [2], 99% of which occurred in developing countries [1]. A global systematic analysis in 2013 attributed 15% of childhood deaths to ALRI, particularly pneumonia [3]. In developing countries especially in Africa, ALRI is the leading cause of morbidity and mortality among children in their first five years of life [4]. Among African children, ALRI accounted for 14% of deaths in 2000 and 16% deaths in 2013 [4].

The burden and causes of ALRI have been well documented in industrialized countries [5]. In most surveys where appropriate diagnostic methods have been used, viruses were the major etiological agents of ALRI in children [6-9]. The most common respiratory viruses are respiratory syncytial virus (RSV), human parainfluenza virus (HPIV), human adenovirus (HAdV), influenza virus (IV), human metapneumovirus (HMPV), human bocavirus (HBoV), human coronavirus (HCoV) and rhinovirus (RV). For example, in a recent comparative and retrospective cohort study of infants hospitalized for ALRI in Sweden, respiratory viruses accounted for 92.6% of ALRI with RSV identified as the major viral agent in 51% of children [10]. However in developing countries especially in Africa, there is a dearth of information about respiratory viruses and their impact on the burden of ALRI. Global meta-analyses reports in 2010 and 2011 have estimated the case fatality ratio (CFR) among hospitalized children younger than five years. Respectively, the yearly estimated CFR for children younger than 5 years in developing countries caused by RSV-associated ALRI was 2.10% and influenza virus-associated ALRI was 2·96% [11, 12]. These estimates were higher compared to the 0.7% and 0.17% respectively reported for developed countries. In a recent study of severe acute respiratory illness (SARI) cases from eight African countries, 33.3% of influenza virus-associated severe acute respiratory infections mortality was observed among children aged 0–4 years [13]. Nonetheless only three countries, Kenya, Madagascar and South Africa tested for other respiratory pathogens besides influenza virus and identified a virus in 59% of cases. Few other studies from sub-Saharan Africa have documented mostly RSV and influenza viruses as frequent cause of viral ALRI in children [14-18].

In Ghana, ALRI such as pneumonia and bronchopneumonia are a main reason for hospitalizations and death among young children [1, 19, 20]. The World Health Organization (WHO) reported that in Ghana, ALRI accounted for 13% of child deaths in 2013 [20]. According to an earlier report by the Ghana Health Service in 2011, a total of 44393 pneumonia cases in children under five years of age were recorded. This resulted in an 11.3% increase in hospital admissions between 2010 and 2011. Moreover, 170 (0.38%) deaths were recorded. Additionally, few other investigations from Ghana have described ALRI with 3.1-7.4% mortality rates among hospitalized children [21-23]. However in these studies, only a limited number of respiratory pathogens were tested, with RSV been the major pathogen identified. The generally scanty information concerning respiratory viruses in Ghana and sub-Saharan Africa at large, is probably due to the weak disease surveillance for overall and cause-specific ARLI hospitalization [17]. However in view of vigorous initiatives towards the development of vaccines and antivirals for prophylaxis and treatment of respiratory virus infections [24, 25], an understanding of the epidemiology and circulation patterns of these infections is needed for optimizing healthcare strategies in developing countries. This necessitates among other measures, the urgent need to improve upon the diagnosis of respiratory infections and surveillance for respiratory viruses, especially in Africa.

1.2 Clinical manifestations of ALRI

In general, respiratory tract infections are distinguished by upper respiratory tract infection affecting the nose, sinuses and throat, and by lower respiratory tract infection (LRTI), affecting the airways and lung (Fig. 1). The main symptom of ALRI is a severe cough bringing up phlegm and mucus. Moreover, pneumonia, bronchopneumonia, bronchiolitis and bronchitis to mention a few, are characteristic for ALRI [26].

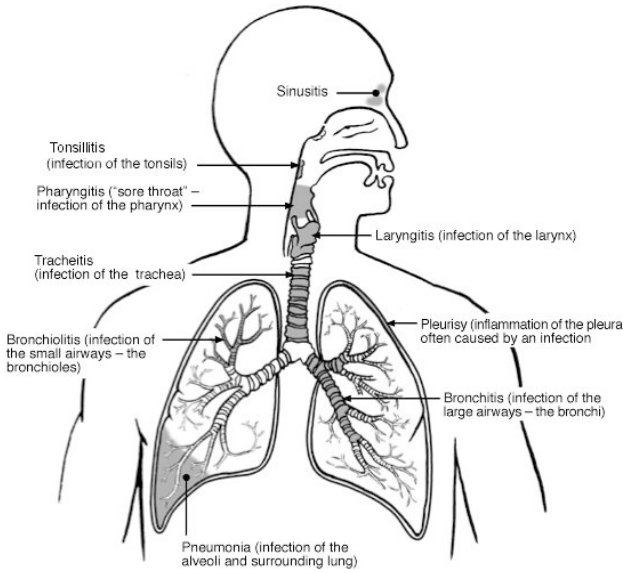


Fig. 1: Infections of the respiratory tract. Source: [27].

1.2.1 Pneumonia and Bronchopneumonia

Pneumonia can be defined as an acute inflammation of the parenchyma of the lower respiratory tract [26]. Bronchopneumonia is a type of pneumonia with patchy consolidation around the larger airways or bronchi.

The definition of pneumonia may vary according to organization, institution or health care setting [28]. The WHO guidelines include a standardized definition of pneumonia based on clinical signs such as a history of cough and/or difficult breathing of less than 3 weeks duration, increased respiratory rate dependent on age, lower chest wall in drawing, cyanosis and/or inability to feed or drink [29]. According to the world statistics report, over 7% of the world's population is affected with pneumonia each year [4]. In 2013, pneumonia contributed significantly to 16% of all under-five childhood deaths worldwide [3]. Rational treatment for pneumonia depends on knowing the most likely pathogens in each community, as the relative frequency of different agents may vary from one geographical region to another [28]. However identifying the causal pathogen in children with ALRI is particularly difficult, as several pathogens including viruses, atypical and typical bacteria may be involved in childhood pneumonia [30]. Pneumonia can sometimes be difficult to diagnose as it shares many signs and symptoms with several other conditions such as chronic obstructive pulmonary disease, asthma, pulmonary edema, bronchiectasis, bronchitis, lung cancer, and pulmonary emboli such as the common cold and asthma [26].

Viruses may act as sole pathogens in pediatric community acquired pneumonia (CAP) or redispose to bacterial pneumonia [31]. Viral pathogens are more common causes of CAP in children younger than 2 years, accounting for 80% of cases [32]. The etiology of viral infestations varies by geography, season, and the age of patients studied. However, RSV, influenza virus, HPIV, HAdV, RV, HBoV and HMPV are described consistently as the most common viruses associated with CAP in children [33]. Viruses, usually as a single cause of pneumonia are less common in older children with the exception of influenza virus [34].
Most respiratory viruses tend to multiply primarily in the epithelium of the upper airway and subsequently infect the lung by means of airway secretions or hematogenous spread [28].

Severe pneumonias may result in extensive consolidation of the lungs with varying degrees of hemorrhage. Some patients showed bloody effusions and diffuse alveolar damage [28]. The mechanism of damage to tissues depends on the virus involved [35]. Viral infections are characterized by the accumulation of mononuclear cells in the submucosa and perivascular space, resulting in partial obstruction of the airway. Some viruses are mainly cytopathic, directly affecting the pneumocytes or the bronchial cells. With others, over exuberant inflammation from the immune response is the mainstay of the pathogenic process [35].

RSV is the commonest cause of viral pneumonia, especially in the first 3 years of life [36]. Approximately 5-40% of pneumonia hospitalizations are due to RSV [12, 37]. High risk groups for severe RSV disease include infants below six months of age, premature infants, children with chronic lung disease, congenital heart disease, immunodeficiency or cystic fibrosis, and infants with neuromuscular diseases [38, 39]. Mortality rates associated with RSV infection are generally lower than 1% in previously healthy infants, but increase significantly up to 73% in high risk children [40]. There is currently no effective treatment or vaccine available for RSV. Prophylactic use of human anti-F monoclonal antibody and palivizumab is recommended in high-risk groups which efficiently reduce the risk of RSV-associated ALRI and hospitalizations [24].

Influenza virus pneumonia is responsible for a substantial morbidity among children. Children usually present with upper respiratory tract infections, but some may develop pneumonia resulting in the need for hospitalization [41]. Studies in the USA demonstrated that influenza-related hospitalizations ranged from 1.9-16.0 per 10,000 children per year [42]. Surveillance data from the European region have also indicated that up to 9.8% of children below 14 years

present to a physician with influenza in an average season [43]. In sub-Saharan Africa, a systematic review showed that up to 15.6% of children admitted to hospital for ALRI had influenza virus identified [44]. Primary influenza virus infections can as well result in severe outcomes such as death [11, 45]. The 2011 meta-analysis by Nair and colleagues estimated that between 28,000 and 111,500 children aged below five years die each year from ALRI associated with influenza virus, and that 99% of deaths happen in developing countries [11].

HPIV cause a spectrum of respiratory illnesses and is second in importance to RSV in causing lower respiratory tract disease in children and pneumonia in infants younger than 6 months [28, 37]. It is estimated that 12% of hospitalizations for LRTI in children are due to HPIV [46]. Pneumonia from HPIV3 infection occurs primarily in the first six months of life [47]. HPIV1 and HPIV3 each cause about 10% of outpatient pneumonias, although HPIV3 causes a larger percentage of infections in hospitalized patients. Pneumonia can be caused by both HPIV-2 and HPIV-4, however the incidence of disease is not well described [47]. HPIV4 affects older children and is the least common type [46]. Studies from the USA reported that HPIV1 and HPIV2 are more frequently associated with laryngotracheobronchitis (croup) [37, 48, 49]. In addition to respiratory illness, children with HPIV infection can significantly presented with diarrhea, seizures, otitis media, rash, red eyes, and hypoxia has reported by several studies [37, 50-52].

HAdV are an important cause of infections in children representing up to 17% of ALRI [53-55]. HAdV cause a wide variety of illnesses including pneumonia in children. In a community setting, HAdV accounts for 10% to 21% of pneumonias in children younger than 5 years of age, and can occur at any time of the year [53]. Different HAdV serotypes have been are associated with adenovirus-induced pneumonia. However serotypes of the species B and C are the most commonly identified in the pneumonia infections [56]. HAdV have also been identified as cause of frequent outbreaks in community settings [57, 58]. Mixed viral-bacterial infection is found in 33% to 66% of CAP cases [59, 60].

1.2.2 Bronchiolitis and bronchitis

Bronchiolitis is an acute infection of the lower respiratory tract causing inflammation of the bronchiolar epithelium with peribronchial infiltration of white blood cell types, mostly mononuclear cells, and edema of the submucosa and adventitia [61]. Acute bronchitis is

defined as inflammation of the bronchial respiratory mucosa resulting in productive cough [28].

Bronchiolitis is the most common LRTI in infants aged 3 to 6 months. It is clinically diagnosed in children presenting with breathing difficulties, cough, poor feeding and irritability, combined together with wheeze and/or crepitations on auscultation [62]. 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. Underlying medical problems such as prematurity, cardiac disease or underlying respiratory disease give more severe disease. In preterm infants less than six months of age, admission rate with acute bronchiolitis is 6.9% with more frequent admission to intensive care unit [61, 62]. The risk of death for a healthy infant with bronchiolitis is less than 0.5%, but the risk is much higher for children with congenital heart disease (3.5%) and chronic lung disease (3.45%) [61]. About 40-50% of hospitalized infants with bronchiolitis proceed to a persistent cough and recurrent viral-induced wheeze, probably related to continuing inflammation and temporary cilial dysfunction [37, 62]. Acute bronchitis and bronchiolitis share many pathological and clinical features, and the same agents may induce both conditions [28].

Bronchiolitis is associated with viral infections. RSV is responsible for 70-75% cases of bronchiolitis [61, 62]. Approximately 70% of all infants will be infected with RSV in their first year of life and 22% develop symptomatic disease. In a study from the UK, RSV-attributed death rate in infants up to aged 12 months was 8.4 per 100,000 populations [62, 63]. In older children and adults, RSV infections usually range from asymptomatic to upper respiratory tract presentations but causes very severe disease in the elderly [40]. RSV induces only partial immunity and re-infections are common throughout life [64-66].

Other viruses involved in bronchiolitis are HPIV1-3, HMPV, RV, HAdV, influenza virus, HBoV, and HCoV [67-70]. HPIV3 is more likely to cause bronchiolitis in approximately two-thirds of children in the first year of life [48, 71]. HMPV has a clinical course similar to RSV and has been estimated to account for 3% to 19% of bronchiolitis cases [72]. Globally HMPV has been linked to acute respiratory illness in individuals of all ages [72]. About 4–16% of ALRI patients are affected with HMPV and 10% may require hospitalization [73]. In the pediatric population, HMPV is commonly found in children less than 2 years old and accounts for 5–15% of hospitalizations [73].

Following RSV, RV is the second most common cause of severe bronchiolitis in hospitalized children. RV accounts for14% of RV-associated LRTI admissions to pediatric intensive care units and has been associated with wheezing illnesses during infancy and exacerbations of asthma among older children with reactive airway disease [68, 74]. Nonetheless, RV is a frequent cause of mild upper respiratory tract infections and has been identified in up to about 40% of asymptomatic persons [75, 76].

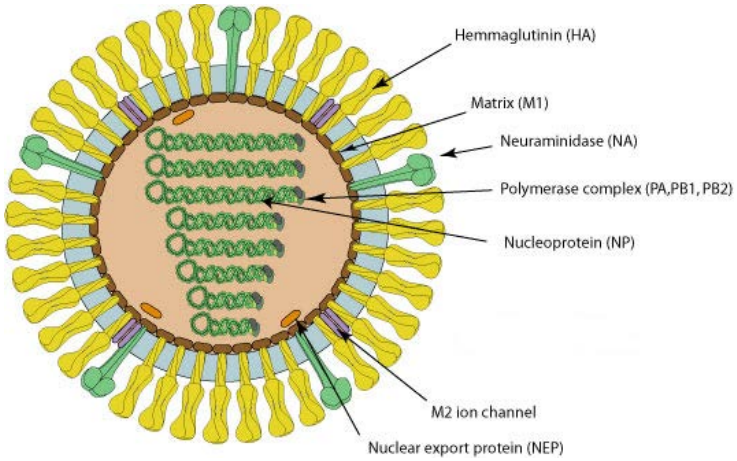
Although HCoV display a wide range of symptoms, reports have suggested that they may have a significant role in pediatric ALRI and hospitalizations [77]. The four HCoV (HCoV-229E, HCoV-HKU1, HCoV-NL63, and HCoV-OC43) are associated with a range of respiratory outcomes including bronchiolitis and pneumonia. Specifically, HCoV-NL63 has been associated with croup [78, 79] and HCoV-HKU1 with febrile convulsion [79, 80]. A clear link between HCoV-NL63 and respiratory diseases was established in the German prospective population-based study on LRTI in children less than 3 years of age [81]. Of the children with HCoV-NL63 infections, 45% had laryngotracheitis (croup) compared to only 6% in the control group. Viral coinfection rates in bronchiolitis ranged from 15% to 42% among hospitalized children; most commonly with RSV and/or HMPV or rhinovirus [82, 83].

1.3 Virology and molecular epidemiology of respiratory viruses

1.3.1 Influenza viruses

Influenza viruses are enveloped RNA viruses belonging to the *Orthomyxoviridae* family [84]. *Influenza virus A, B and C* represent three of the five genera within the family. The virion is usually rounded but can be pleomorphic, ranging from 80-120nm in diameter (Fig. 2). Influenza virus has a linear, segmented, single-stranded, negative-sense genome with a total size of 10-14.6kb [84]. Within the viral core are eight viral RNA segments for influenza virus A and B, and seven segments for influenza virus C [85]. Segment lengths range from 736-2396nt, basically encoding for polymerase protein (PB1, PB2 and PA), nucleoprotein (NP), Matrix protein (M) as well as the non-structural protein/nuclear export protein (NS/NEP). Inserted in the lipid membrane are the hemagglutinin (HA) and neuraminidase (NA) protein [85].

(a)



(b)

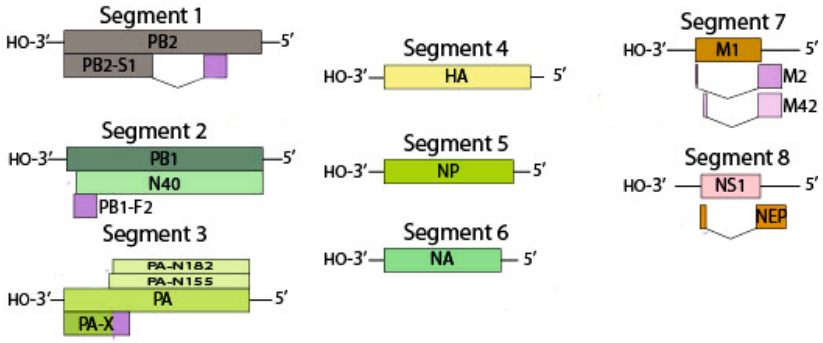


Fig. 2: Schematic structure of influenza virus (a) Virion and (b) Genome. Adapted from [85].

1.3.1.1 Genetic variability of influenza viruses

Influenza virus genome encodes 12-14 proteins depending on strains; PB2, PB2-S1, PB1, PB1-F2, PA, PA-X, HA, NP, NA, M1, M2, M42, NS1 and NEP [84, 85]. The M1 protein forms a shell which gives strength and rigidity to the lipid envelope, High level of M1 protein also induces genomes segments export from nucleus by NEP protein. M2 protein functions as ion channels and is used as target for some antiviral drugs. The HA and NA proteins determine the subtype of the virus and are important targets of antibodies against the virus. However primarily, antigenic variation of the HA is responsible for the immune escape from the human host. Currently 18 HA and 11 NA subtypes of influenza A virus are known [86]. Subtypes H1, H3, N1 and N2 presently circulate in the human population [87, 88]. H2 only corresponded to a major pandemic during 1957 to 1968. H5, H7 and H9 subtypes cause sporadic transmissions from infected poultry. Influenza B viruses are divided into two

antigenically diverged lineages: B/Victoria/2/1987-like and B/Yamagata/16/1988-like viruses [89]. The B virus primarily infects humans, but has also been isolated occasionally from seals.

Antigenic drift and shift are the mechanisms by which influenza A viruses change their antigenic properties [90]. Antigenic drift occurs through continuous mutation of the RNA genome of the virus. Antigenic shift arise from major genetic changes in the HA and/or NA proteins and may result in an entirely new influenza A virus subtype in the human population. The shift may form the scenario for a pandemic outbreak if efficient human-to-human transmission occurs [90]. Alternatively a genetic reassortment may occur between human influenza virus and avian or pig influenza virus and might result in the formation of a new virus to which the population is immunologically naïve [87].
In the last century, four influenza virus epidemics were documented; the 1918 Spanish flu (H1N1), 1957 Asian flu (H2N2), 1968 Hong Kong flu (H3N2) and the 1977 Russian flu (H1N1) [87, 90]. In 2009, a novel influenza A (H1N1) virus caused another pandemic [91]. The influenza A(H1N1)pdm09 virus was determined to be a triple influenza virus variant of swine, avian and human origin [90, 91].

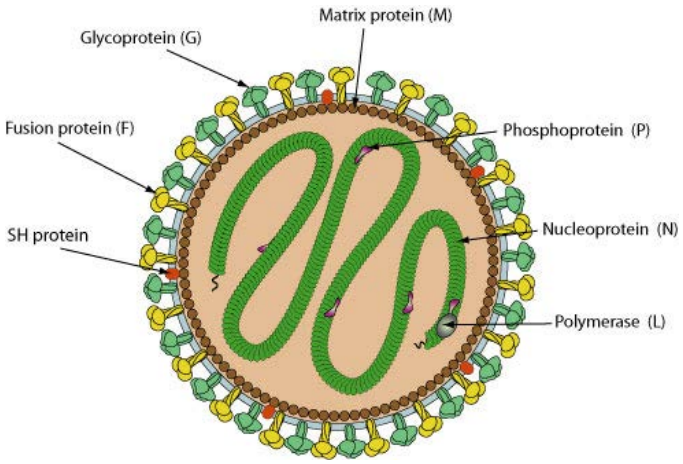
1.3.2 Respiratory syncytial virus

Respiratory syncytial virus (RSV) is an enveloped virus that belongs to the family *Paramyxoviridae*, subfamily *Pneumovirinae* within the genus *Pneumovirus* [84]. The viral particle is asymmetrically spherical in shape with a diameter of about 150nm (Fig. 3). The virus has a negative-sense single-stranded RNA genome of 15,191-15,226nt [84]. The genome contains ten genes encoding for 11 proteins [92, 93]. The viral ribonucleoprotein (RNP) consists of the RNA genome encapsidated by the nucleoprotein (N), phosphoprotein (P) and RNA-dependent RNA polymerase (L) as well as the M2-1 protein. The viral genome also encodes the structural matrix protein (M), Matrix M2-2 protein and three transmembrane surface proteins; the small hydrophobic glycoprotein (SH), the attachment glycoprotein (G) and the fusion glycoprotein (F) [93].

Upon accumulation of viral proteins, the viral polymerase switches from transcription to viral replication, a process thought to be controlled by M2-2 [92]. The F protein is required for fusion between the cellular and viral membranes. The G protein is involved in host cell attachment [92]. Epitopes on the G and F glycoproteins are targets of neutralizing antibodies

[65, 94]. However only the G-protein is known to accumulate mutations in response to host immunological pressures and thus used for genotyping of the virus.

(a)



(b)

Fig. 3: Schematic structure of Pneumovirus (a) Virion and (b) Genome. Adapted from [93].

1.3.2.1 Molecular epidemiology of RSV

Two groups of RSV, A and B are known to exist based on the antigenic [95] and genetic [96] variability of the virus. Comparisons among RSV group A and B revealed extensive glycoprotein G gene differences between the groups and relative conservation within the groups [96]. The two groups A and B were individually very similar (2-6% amino acid differences), whereas between the two groups wide diversity (44-47% amino acid differences) was observed. The intra-group genetic diversity showed amino acid differences of up to 20% among the group A, and 12% among the group B viruses [96]. The variability in the G protein gene is linked to poorly conserved amino acid motifs at potential glycosylation sites which are most prone to substitutions in both RSV group A and B [96, 97]. In a recent study, the predicted N-glycosylation at certain sites of the G protein displayed evidence of diversifying selection for the RSV strains. These sites included amino acid positions 160, 187, 224, 226, 230, and 239 [98]. Additionally, the total number of predicted O-glycosylation sites in the G protein was 36% higher for RSV group B strains than for group A strains. Amino acid site alterations that are correlated with either changes in N- and O-glycosylation potential or susceptibility to antibody neutralization can trigger RSV phenotypic differences [98].

The genetic variability of the G protein also provided an opportunity to better define the epidemiology of these viruses [64]. Several RSV genotypes have been characterized based mainly on the second hypervariable region (VR2) of the G gene ectodomain [99]. Presently there are 15 RSV group A genotypes found to have circulated. These include GA1-GA7 [64, 94], SAA1 [100], SAA2 [101], NA1-2 [102], NA3-4 [103] CB-A [104] and ON1 [105].

The ON1 was first identified in 2010 from Canada as a 'novel' RSV group A genotype due to its 72-nucleotide duplication in the VR2 of the G protein gene [105]. This duplication resulted in codon disruption and lengthening of the subsequent predicted polypeptide by 24 amino acids, including 23 duplicated amino acids. Three unique substitutions E232G, T253K and P314L were noted to be specific for ON1 genotype. Dissemination of the 'novel' ON1 genotype in several countries has been described [101, 106-108]. The circulation of the 'novel' ON1 genotype was initially reported as a non-dominant genotype in 1-10% of the RSV population in Canada [105], South Africa [101], Thailand [108], India [109], Japan [107], China [110] and Malaysia [111]. More recent data from Kenya [112], South Korea [113], The Philippines [114], Germany [106], Italy [115] and Spain [116] showed that ON1 genotype was rapidly spreading as the dominant RSV-A genotype in 62-94% of the RSV population. Since the NA1 genotype was first identified in Japan, it has been reported as the majority RSV-A genotype from 2006 to 2012. The reports included long-term studies from South Africa [101], and mostly Asian countries such as Cambodia [117], Japan [118], China [103, 110] and the Philippines [119]. The SAA2 genotype emerged in 2006-2007 from a retrospective study in South Africa; it has rarely been reported by others. However earlier investigations prior to 2012 demonstrated a stable circulation of GA2 and GA5 and sporadic circulation of GA7, with different patterns of dominance [66, 102, 120].

Similarly a number of genotypes have been described for RSV group B including GB1-4 [64, 94], SAB1-3 [100], SAB4 [117], URU1-2 [121], THB [108], BA [122], BA1-6 [123], BA7-10 [124], BA11 [104], CB-B [104], GB1-13 [65, 120], BA12 [111], BA-C [103], CB-1 [103], and GB5 [125]. Nonetheless much overlap has been observed among the RSV group B genotypes, making it difficult to distinguish between them. For example CB1, THB and GB5 strains classified differently by different studies are the same genotype and show identical sequences of the VR2 of the G protein gene. GB13 are the same as BA1-6, BA4 is intermixed with BA7-10, and BA-C is closely related to BA3.

Earlier in 1999 from Argentina, the BA genotype with its 60-nucleotide duplication in the same VR2 of the G protein was identified as 'novel' by Trento et al [122]. The BA genotype

subsequently progressed from novelty to become dominant worldwide, largely replacing formerly circulating RSV-B genotypes [65, 126, 127]. Accumulation of nucleotide changes was shown to result in sequence variation within the 60-nt duplication overtime [128]. In a study by Trento et al, later BA viruses from 1999 were closely related to the putative ancestor BA virus, and contained an exact copy of the duplicated segment [123]. However, further genetic drifts of viruses in successive epidemics resulted in new antigenic groups [123]. Additionally many other studies have subdivided the BA genotypes into several genotypes, and up to now, 13 different BA (BA1-13) genotypes have been described [100, 120, 124]. It was hypothesized that several amino acid substitutions located in the VR2 led to enhanced viral fitness and replacement of the original BA viruses [120].

In line with the BA genotype rapid spread from novelty to dominance, the ON1 genotype may presumably result in a similar selection advantage to replace other existing RSV-A genotypes [105]. A recent analysis of global data from 21 countries suggested that the ON1 genotype is evolving and has disseminated worldwide with different lineages [129].
RSV has a worldwide distribution and shows clear seasonality. In temperate climates, outbreaks occur yearly in the late fall, winter, or spring but not in the summer [66, 94, 130]. In tropical and subtropical regions, epidemics occur usually during the rainy period [131-133]. Both RSV groups can be present in the same community and their relative proportions may differ between epidemics although group A viruses tend to predominate [66, 101]. Moreover, several genotypes can co-circulate in a single epidemic season, and different genotypes can dominate in consecutive seasons [64, 100, 120].

1.3.3 Human metapneumovirus

Human metapneumovirus (HMPV) is classified as a member of the genus *Metapneumovirus* of the *Pneumovirinae* subfamily within the *Paramyxoviridae* family [84]. The viral particles are enveloped, pleomorphic spheres and filaments, similar to RSV virion (Fig. 3). The genome is a single-stranded negative-sense RNA of 13,280-13,378nt, and comprise of nucleoprotein (N), phosphoprotein (P), matrix protein (M), fusion protein (F), matrix proteins (M2-1 and M2-2), small hydrophobic protein (SH), glycoprotein (G) and RNA-dependent RNA polymerase (L) genes. The M2 gene contains two open reading frames and encodes the M2-1 and M2-2 proteins. The eight-gene RNA genome codes for nine different proteins [84].

1.3.3.1 Molecular epidemiology of HMPV

Based on genomic sequencing and phylogenetic analysis, there are two major subgroups of HMPV, designated A and B [134, 135]. The major differences between the A and B subgroups are nucleotide polymorphisms in the G and SH proteins. Nucleotide variability in the G protein resulted in significant amino acid variability in the extracellular domain of the protein. An overall 32-37% amino acid identity of the G protein between the A and B genotypes of HMPV was reported [136, 137]. However among HMPV genes, the F gene sequence is relatively highly conserved [135, 136, 138]. The sequence conservation was postulated to be a major determinant for cross-lineage neutralization and antibody response. The F gene homology within each subgroup showed sequence identity of 94.3–100% and 98.3–100% respectively, at the nucleotide and amino acid levels [139]. Between the subgroups, nucleotide and amino acid identity was 83.0–83.6% and 94.1–95.4% respectively. The amino acid identity is more conserved than nucleotide identity, suggesting structural or functional constraints on F protein diversity [140].

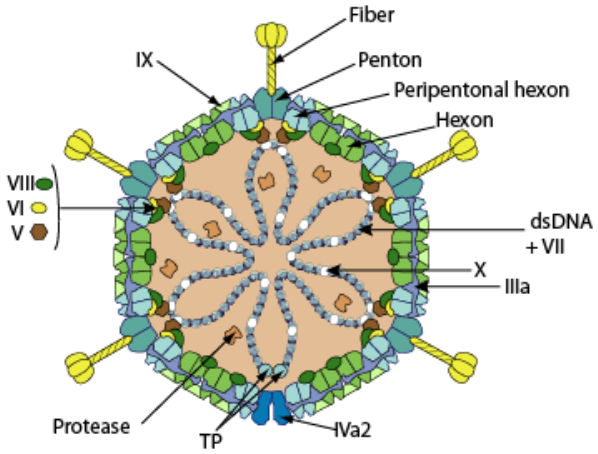
The HMPV subgroups have been further classified into four genetic lineages A1, A2, B1 and B2 and two sub-lineages A2a and A2b [140-142]. Intergenotypic comparison of the amino acid sequence of the F gene identified a number of conserved amino acid residues specific for each subgroup or lineage [142]. HMPV is distributed worldwide and has a seasonal distribution comparable to that of RSV [143]. In temperate regions, epidemics tend to strike in the late winter and early spring [144-146], whereas in the tropics or subtropics HMPV epidemics peaked in spring and summer or during wet seasons [147-150]. Studies have found that both subgroups may co-circulate simultaneously but during an epidemic, one subgroup usually dominates [73, 143].

1.3.4 Human Adenoviruses

Human adenoviruses (HAdV) belong to the genus *Mastadenovirus* of the family *Adenoviridae* [84]. The viral particles are structurally icosahedral, non-enveloped, double-stranded, linear DNA viruses with a diameter of 70-100nm (Fig. 4). The genome sizes range from 26,163-48,395nt. The central part of the genome is well conserved, whereas the two ends show large variations in length and gene content from other members of the family. About 40 different polypeptides including the hexon, fiber and penton base proteins are produced mostly via complex splicing mechanisms [84]. Virus entry occurs by attachment

through the fiber knob proteins, and subsequent internalization through the penton base proteins [151]. The hexon proteins are involved in neutralization. These antigens at the surface of the virion are mainly used for differentiation of the virus into serotypes and types, by hemagglutination-inhibition assays and genomic sequencing [84, 151].

(a)



(b)

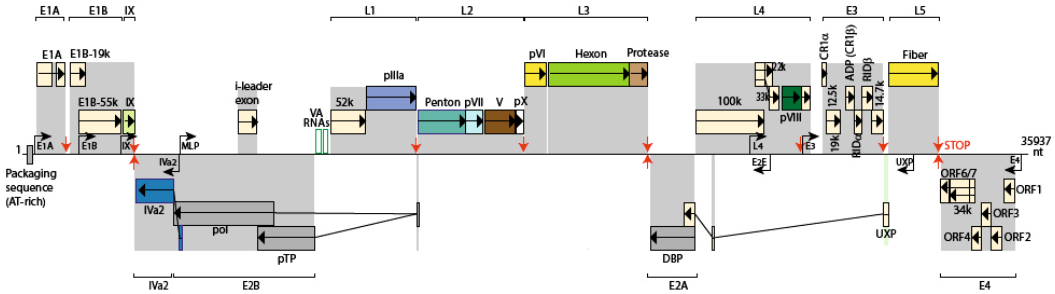


Fig. 4: Schematic structure of HAdV (a) Virion and (b) genome. Adapted from [152].

1.3.4.1 Molecular epidemiology of HAdV

HAdV are classified into the seven species A to G with further subdivision of species B into subspecies B1 and B2 [84]. The viruses belonging to individual HAdV species display high similarity to each other at the nucleotide level and do not commonly recombine with members of other species [151]. Currently more than 67 HAdV types have been published; types 1 to 51 were characterized by serotyping. The remaining HAdV types identified since 2007, were detected by genomic and bioinformatics analyses [153, 154].

Homologous recombination and mutation are important evolutionary processes driving genetic variation within HAdV genomes [155]. HAdV recombination events can result into

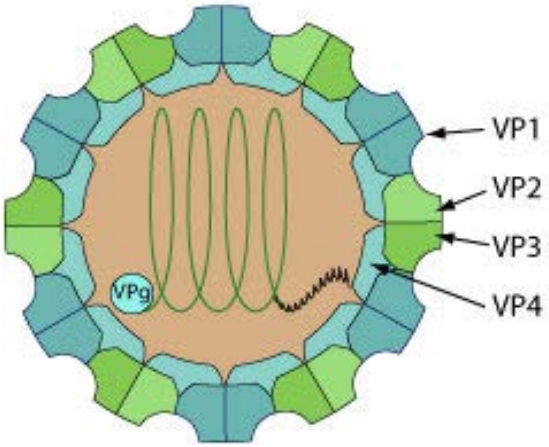
new viruses displaying different tissue tropisms and increased virulence. The majority of novel HAdV types identified by genomic analysis belong to species D, and they were shown to include sequences derived from multiple other types from the same species [155]. For example, HAdV-D53 resulted from recombination in the penton-base, hexon, and fiber regions of HAdV-D22, D37, and D8, respectively [156]. In a recent study by Chen et al, HAdV strains having penton-base gene of HAdV-1, and hexon gene and fiber gene of HAdV-2 were identified from patients with acute respiratory disease (ARD), indicating intraspecies recombination [157]. Similarly in another study, the occurrence of a new recombinant strain between HAdV-7 hexon gene and HAdV-3 fiber gene was associated with fatal outcomes during an outbreak of acute respiratory infections (ARI) among infants in Portugal [158].

The grouping of HAdV into different species reflects, in part, the general cell tropism of the viruses and the resulting diseases and symptoms [159]. HAdV-A is commonly associated with meningoencephalitis; HAdV-B with meningoencephalitis, pneumonia, cystitis and keratoconjunctivitis; HAdV-C with pneumonia and hepatitis; HAdV-D with meningoencephalitis and keratoconjunctivitis; HAdV-E with pneumonia, and HAdV-F and G are commonly associated with gastroenteritis. However, other HAdV species may also occur at the indicated sites of infection [151]. The HAdV types most commonly reported to be associated with respiratory disease worldwide are HAdV-C1, C2, C5, B3, B7, B21, E4, and F41 (20, 62–66). For example, in long-term studies from Thailand [160], Egypt [161] China [162] and Argentina [163], HAdV types 1-7 accounted for the majority of all adenoviral infections observed. HAdV types 3, 4 and 7 have often been found to cause outbreaks in communities [57, 58, 164]. Most HAdV species appear to circulate globally, but predominant types differ between countries and geographic regions. HAdV predominant types may change over time as demonstrated in reports from Thailand [160] , Peru [55] and Malaysia [165] and Korea [166].

1.3.5 Human rhinoviruses

RV are members of the genus *Enterovirus* within the family *Picornaviridae* [84]. RV is a linear, positive-sense, single-stranded RNA virus of approximately 7,200-8,500nt. The virions are non-enveloped, spherical and about 30nm in diameter (Fig. 5). The genome consists of a single gene whose translated protein is cleaved by virally encoded proteases to produce 11 proteins [167]. Four proteins, VP1, VP2, VP3, and VP4 makeup the viral capsid that encases the naked RNA genome, while the remaining nonstructural proteins are involved in viral genome replication and assembly. The VP1, VP2, and VP3 proteins account for the virus' antigenic diversity, while VP4 is located on the internal side of the capsid and anchors the RNA core to the capsid [167]. There are 60 copies each of the four capsid proteins giving the virion an icosahedral structure with a canyon in VP1 that serves as the site of attachment to cell surface receptors.

(a)



(b)

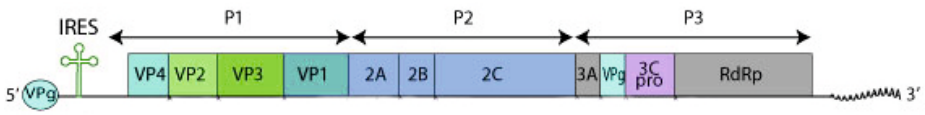


Fig. 5: Schematic structure of RV (a) Virion and (b) Genome. Adapted from [168].

1.3.5.1 Molecular epidemiology of RV

RV are classified into three species; A, B and C, based on phylogenetic sequence criteria [84]. Each RV species shows remarkable genetic and or antigenic heterogeneity [167]. RV species A and B were characterized by cross-neutralization assays and genome sequencing into 100

serotypes; species A (75 serotypes) and species B (25 serotypes) [169]. The RV species C has only recently been recognized by genomic sequencing [170].

Recent classification of RV into genotypically assigned types has been based on new proposed criteria [171, 172]. Sequences from the capsid genes, VP1 and partial VP4/VP2 show evidence for marked phylogenetic clustering. A newly identified RV type should be phylogenetically distinct from all previously classified types. In addition, new types should demonstrate an indicative VP1 and/or VP4/VP2 divergence threshold specified for their respective species [171, 172]. Classification on the basis of VP4/VP2 sequence divergence alone is a provisionally assigned type (PAT) until a matching VP1 sequence is obtained. Presently there are 77 RV-A types and 4 RV-A PATs, 29 RV-B types and 4 RV-B PATs, and at least 51 RV-C types and 14 RV-C PATs [171].

RV are distributed worldwide. In temperate countries, infections occur primarily in two peaks, the first in spring and the second in early autumn [173-175]. In tropical, subtropical and semiarid regions, RV show a possible seasonality peak during the rainy season [176, 177]. Most RV infections during peak activity have been attributed to RV species C.

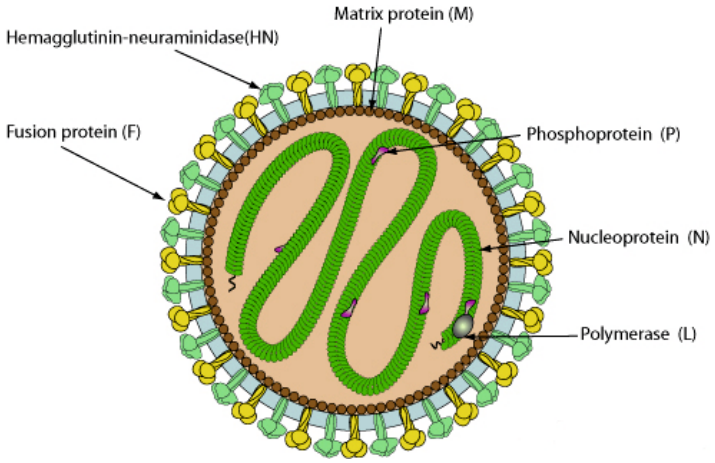
Some studies suggested that RV species C cause more severe respiratory illness than RV species A or B [174, 178]. In a study from the Philippines, RV viremia was reported in 31% of children with RV-C infection, compared to 3% and 0% of children with RV species A and B infections respectively [179]. Other studies however, found no difference in disease severity among RV species [175, 176, 180, 181]. RV species B are generally considered to be rare, with an average prevalence rate of about 7% among RV infections [182].

1.3.6 Human Parainfluenza viruses

Human parainfluenza viruses (HPIV) belong to the subfamily *Paramyxovirinae* within the family *Paramyxoviridae* [84]. HPIV comprise of four types; HPIV1-4. HPIV1 and HPIV3 belong to genus Respirovirus, while HPIV2, and HPIV4a and 4b belong to genus Rubulavirus. The virions are enveloped, non-segmented negative-strand RNA of 150nm or more in diameter (Fig. 6). The genomes of HPIV1, 2, and 3 are similar in size (15,462-15,6654nt), whereas that of HPIV4 is somewhat larger (17,400nt) [84, 183]. The genome encodes six structural protein genes in the order: 3'-N-P-M-F-HN-L, which are transcribed sequentially into separate mRNAs, encoding 7–10 proteins. There are two viral surface proteins: The hemagglutinin-neuraminidase (HN) protein mediates attachment to host cell

membranes, and the fusion (F) protein mediates fusion of the viral envelope with host cell membrane. The nucleocapsid (N) protein coats the genomic RNA. Phosphoprotein (P) and the large polymerase protein (L) are associated with the nucleocapsid, while matrix protein (M) coats the inner surface of the envelope. The P gene also encodes additional proteins that vary among viruses. These are called accessory proteins because they are not essential for virus replication *in vitro* [84].

(a)



(b)

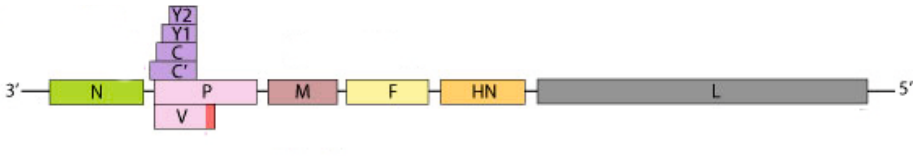


Fig. 6: Schematic structure of HPIV (a) Virion and (b) Genome. Adapted from [184].

1.3.6.1 Molecular epidemiology of HPIV

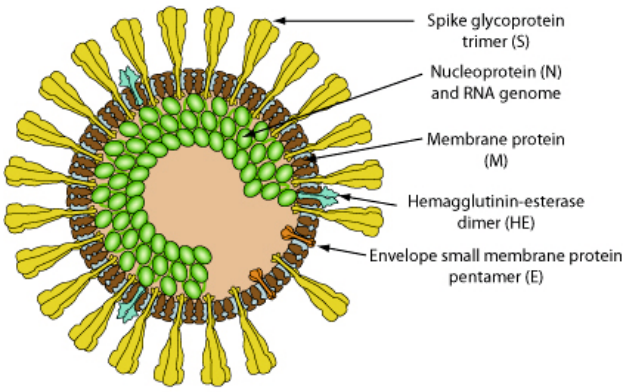
HPIV have a worldwide distribution. Epidemics are known to occur, particularly with HPIV1 [37]. Previous studies have predominantly focused on HPIV1-3 infection because of the high positivity rate and morbidity among children [48, 49, 185]. Several studies have documented distinct temporal trends for HPIV1-3. In studies from China [50, 71], seasonal peaks of HPIV were mostly driven by HPIV3 and in Korea [186] HPIV1 was the predominant type. Biennial fall epidemics of HPIV1 have been reported in previous studies from the US [49, 187]. HPIV2 was reported to also cause infections biennially with HPIV1 in alternating years or yearly outbreaks. Only few studies have reported on the epidemiology of HPIV4 and the

infection rates were quite too low to clearly identify seasonal peaks and activity [50, 187, 188]. However a 4-year retrospective chart review study from the US reported a year-round prevalence with biennial peaks in odd-numbered years for HPIV4 [51]. Most seasonal associations of HPIV were described in temperate areas, whereas studies in tropical regions such associations are not widely defined. In a study from Brazil, HPIV3 was shown to correlation to the dry season with higher activity observed from September to November [185]. Different geographic locations may lead to different seasonal distributions of HPIV types.

1.3.7 Human Coronaviruses

Human Coronaviruses (HCoV) are enveloped RNA viruses which belong to the family *Coronaviridae* and subfamily *Coronavirinae* [84]. HCoV are positive-sense, single-stranded RNA viruses of about 120-160nm in diameter (Fig. 7). The genome sizes range from 26,400-31,700nt. HCoV all encode 15-16 replicase related proteins, 4-5 structural proteins and 1-8 group-specific or accessory proteins [189]. The structural proteins are the spike protein S, which is a class I fusion protein that mediates receptor-binding and membrane fusion [84]. The membrane glycoprotein (M) is believed to associate with the inner leaflet of the membrane to form a matrix-like lattice, responsible for the remarkable thickness of the coronavirus. The envelope protein (E) plays a role in virion assembly and morphogenesis. The nucleocapsid protein (N) is involved in genome encapsidation, RNA synthesis and translation [84]. Many of the replicase proteins are assembled into replication machinery in double membrane vesicles and on a reticular network of membranes that are derived from the endoplasmic reticulum [189].

(a)



(b)

Fig. 7: Schematic structure of HCoV (a) Virion and (b) Genome. Adapted from [190].

1.3.7.1 Molecular epidemiology of HCoV

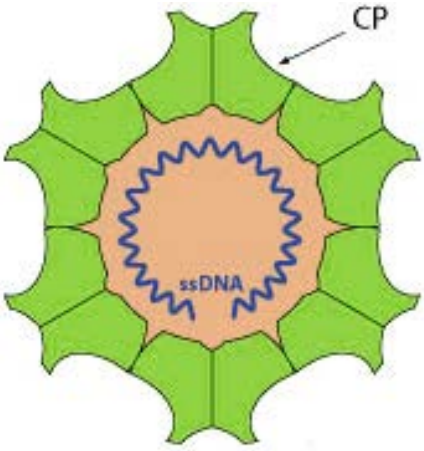
HCoV-NL63 and HCoV-229E belong to the *alphacoronaviruses*. HCoV-OC43 and HCoV-HKU1 are members of the *betacoronaviruses* [191]. These groups were originally designed on serological reactivity, suggesting that antibodies could cross-react with the other virus from the same group. Antibodies directed to the spike protein have the potential to be neutralizing, and in case these antibodies cross-react, seroconversion towards one HCoV might protect against infection by the other virus from the same group [192]. Severe acute respiratory syndrome coronavirus and the Middle East respiratory syndrome coronavirus are members of the *Betacoronaviruses* [191].

HCoV have a global distribution, although there are differences in the frequency of detection of the four viruses in different parts of the world at different times [193]. HCoV-OC43 and NL63 are the most frequently detected species. In temperate regions OC43 tend to emerge in fall and peak in winter whereas NL63 tended to emerge in winter and peak in spring [78, 79, 191]. HKU1 and 229E are uncommon and emerge during winter months. In the tropics and subtropics only few reports have described HCoV species circulation, but a general seasonality is not defined [80, 194-196].

1.3.8 Human Bocavirus

Human Bocavirus (HBoV) belong to the genus *Bocaparvovirus*, within the subfamily *Parvovirinae* and family *Parvoviridae* [84]. The virus is non-enveloped, has an icosahedral symmetry and a diameter of 21-22nm (Fig. 9). The capsid (CP) consists of 60 copies of CP protein. The genome is a linear single-stranded positive and negative-sense DNA of about 5.5kb in size. Three open reading frames (ORFs) located on the DNA strand encodes two non-structural proteins (NS1 and NP1) and the two structural viral capsid proteins (VP1 and VP2). VP2 is translated by leaky scanning from VP1 gene, VP3 would be produced by cleavage of VP2.

(a)



(b)

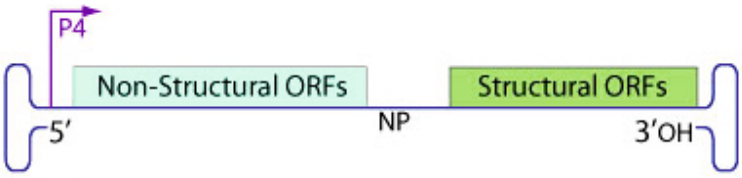


Fig. 8: Schematic structure of HBoV (a) Virion and (b) Genome. Adapted from [197].

1.3.8.1 Epidemiology of HBoV

HBoV was first reported in 2005 in Sweden, from pools of nasopharyngeal aspirates obtained from individuals with respiratory tract infections [198]. Study reports have since then indicated prevalence rates of between 1.5-24.6% in respiratory samples of children [199-201]. HBoV circulates worldwide and currently have been classified into four genotypes, HBoV1-4, based on their genetic variability [202]. HBoV1 is predominantly a respiratory virus [203, 204], but have also been identified in children with acute gastroenteritis [205]. HBoV2-4 seem to occur mainly in stool samples from children with gastroenteritis [206, 207], but rarely also from respiratory tract specimens. In a study report from Japan, enteric HBoV2-4 pathogens were detected in less than 1% of patients with respiratory tract infections [208]. Similarly in an investigation from China, HBoV2 was identified in 4.3% of hospitalized children less than three years old with ARI [209].

HBoV-positive patients have a high coinfection rate of up to 83% in respiratory samples [210]. In a prospective study of infants and toddlers attending daycare centers in the US, another virus was detected in 72% HBoV-positive cases [211]. In addition, HBoV was more common in illnesses with greater severity; but the detection of HBoV was not associated with the presence of respiratory illness [211]. Some studies which show high HBoV1 detection rates among asymptomatic subjects indicate that HBoV may exist in the respiratory or gastrointestinal tracts as a bystander without causality to the current symptoms [212, 213]. On the other hand, several studies of children and/or adults including asymptomatic controls have shown an association between presence of the virus and symptomatic illness [214-218]. In studies from Italy [219], Norway [220], Korea [221] and China [201, 203], a positive correlation was seen between respiratory illness and high copy numbers of HBoV1 DNA or the presence of HBoV1 monoinfection. This evidence strongly suggests that HBoV is an important respiratory pathogen in children.

HBoV infection is detected throughout the year, but with peaks during winter and early spring season in temperate climate [201, 222]. In tropical regions, only few studies have investigated HBoV. In two of such investigations from Cambodia [199] and Thailand [215], HBoV infections were detected year-round, with no clear seasonality.

1.4 Aim of the study

ALRI are a leading cause of morbidity and mortality in children from developing countries [1]. In sub-Saharan Africa and particularly in Ghana; ALRI, malaria, diarrheal diseases, malnutrition and parasitic infections are major causes of death among young children [19, 20]. An earlier study report from a rural community in Ghana indicated that, ALRI accounted for 28% of all illness in children less than five years [223, 224]. In 2011, a case fatality rate of 0.38% was recorded among hospitalization due to pneumonia in children aged below five years [19]. However, documentation of the ALRI were based on clinical observations only; with no identification of the etiological agents involved.

Primarily from industrialized countries, respiratory viruses have been associated with ALRI [7, 8]. However, in developing countries and especially in Africa such information is sparse. For instance, there are relatively few studies of the viral etiology of ALRI from neighboring countries like Senegal [18], Nigeria [225], Burkina Faso [226] and Niger [227] where specific viruses were identified in association with ARD in children. In Ghana, collection and analysis of surveillance data is rarely performed and/or limited to a few pathogens. A study comprising 108 Ghanaian children with ALRI in 2001 investigated RSV and bacterial agents, and 18% of the infections was due to RSV [21]. A similar study in 2008 analyzed a number of viral agents including HAdV, RSV, influenza A and B virus, HPIV1-3, as well as bacterial agents. Among the 128 hospitalized children surveyed, a respiratory virus was identified in 25.8% of patients, and RSV prevailed in 14% of cases [22]. Both studies demonstrated a high burden of ALRI, however, the overall prevalence of respiratory viruses may be an underestimation as limited number of respiratory viruses was investigated. A comprehensive viral etiology of ALRI in children in Ghana and the molecular epidemiology of the concerned respiratory viruses still remain largely unknown.

This study aimed to investigate the molecular epidemiology of common respiratory viruses affecting children under 5 years with ALRI in Ghana. The viruses include influenza A and B viruses, RSV group A and B, HMPV, HAdV, RV, HPIV types 1 to 4, HCoV types 229E, NL63, OC43 and HKU1, and HBoV. The Korle-Bu Teaching Hospital (KBTH) and the Princess Marie Louise Children's Hospital (PMLCH) both located in Accra, Ghana, were selected as study sites for enrollment of patients. The study focuses to firstly, determine the prevalence rates of respiratory viruses among children with ALRI. Secondly, describe the association of the respiratory viruses with specific clinical manifestations of ALRI. Thirdly,

define the distribution pattern of respiratory viruses circulating during the sample collection period in Ghana. Lastly and most importantly, to investigate the genetic variability and diversity of influenza A and B viruses, RSV group A and B, HMPV, HAdV and RV identified during the study period.

2 Materials and methods

2.1 Material

2.1.1 Technical equipment and disposable material

Equipment	Type	Source
Biosafety cabinet	1300 Series A2 Class II	Thermoscientific, Hennigsdorf, Germany
	Safe 2020 Class II	
Incubators	Microbiological incubators	Thermoscientific, Darmstadt, Germany
PCR Workstation	In-house production, RKI	Robert Koch Institute, Berlin, Germany
Microscope (Inverted)	SIP 44347, SIP 44348	Zeiss, Jena, Germany
Refrigerator	4-8°C	Bosch, Denham, UK
Freezer	ProfiLine, -20 °C	LiebHerr, Ochsenhausen, Germany
	Forma 88000 series, -80 °C	Thermoscientific, Hennigsdorf, Germany
Pipettor	Single: 100µl, 200µl, 1000µl, 1-5ml	Eppendorf Research® plus, Wesseling-Berzdorf, Germany
	Multichannel: 10µl, 50µl, 300µl	
Centrifuge	Heraeus Fresco 21 refrigerated microcentrifuge	Thermoscientific, Hennigsdorf, Germany Eppendorf
	Centrifuge 5424 R/Minispin® plus	Neolab-Behr Labor-Technik, Heidelberg, Germany
	Plate centrifuge - PerfectSpin P	VWR, Darmstadt, Germany
Heating block	BioShake iQ thermal mixer	Quantifoil Instruments GmbH, Jena Germany
Vortexer	Genie 2, 120V (Model G560)	Scientific Industries, Karlsruhe Germany
Themocyclers	Biometra T300 cycler	Biometra GmbH, Göttingen, Germany
	Mastercycler epGradient cycler S	Eppendorf AG, Hamburg, Germany
Light cycler	480 Instrument II 25032	Roche, Berlin, Germany
Gel documentation	BioDocAnalyze System (Biometra)	Analytik Jena AG, Jena, Germany
Sequencer	3130xl Genetic Analyzer	Applied Biosystems, Foster City, USA
Biometra power pack	P25	Analytik Jena AG, Jena, Germany
Disposable material	**Description**	**Source**
Cell culture flasks	25 cm^2, 75 cm^2	TPP-Sigma-Aldrich Chemie GmbH, Munich, Germany
Cell culture tubes	16 x 25mm style	BD Falcon-Fisher Scientific GmbH, Schwerte, Germany
Cell culture plates	96-well	TPP-Sigma-Aldrich Chemie GmbH, Munich, Germany
Cell scraper	240mm	TPP-Sigma-Aldrich Chemie GmbH, Munich, Germany
Pipettes	1ml, 2ml, 5ml, 10ml, 25ml	BD Falcon-Fisher Scientific GmbH, Schwerte, Germany
Pipette tips (filtered)	10 µL, 100 µL, 1,000 µL)	Thermo Fisher Scientific GmbH, Schwerte, Germany
Cryovials	1.8ml, screw-cap	Thermo Fisher Scientific GmbH, Schwerte, Germany
Sterile filter	0.22 µm, 0.45 µm	Millipore, Billerica, USA
Parafilm	PARAFILM® M sealing filmL × W 15 m × 500 mm	TPP-Sigma-Aldrich Chemie GmbH, Munich, Germany
PCR reaction tubes / Strips	0.2 ml, 0.5ml, 1ml, 8-strips	BioScience-Greiner Bio-One GmbH, Frickenhausen, Germany

Reaction tubes	0.5, 1.5 mL; 2.0 mL	Eppendorf AG, Hamburg, Germany
Cycler plates and sealers	LightCycler® 480 Multiwell Plate 96, white, with sealing foils	Roche Diagnostics Deutschland GmbH, Mannheim, Germany

2.1.2 Chemicals and enzymes

Chemical	Source
Ethanol (96 %)	Merck-VWR International GmbH, Dresden, Germany
Agarose (Ultrapure)	Invitrogen- Thermo Fisher Scientific GmbH, Schwerte, Germany
Bovine serum albumin (BSA)	PAA Laboratories GmbH- VWR International GmbH, Dresden, Germany
Dithiothreolin (DTT)	Sigma-Aldrich, Munich
dNTP/dNUTPs	Invitrogen- Thermo Fisher Scientific GmbH, Schwerte, Germany
GelRed®	Genaxon bioscience-Diagonal GmbH & Co. KG, Münster, Germany
Fetal calf serum (FCS)	PAN-Biotech GmbH, Aidenbach, Germany
Magnesium chloride ($MgCl_2$)	Merck-VWR International GmbH, Dresden, Germany
Turkey erythrocytes	Preclinics GmbH, Postdam, Germany
Guinea pig erythrocytes	Robert Koch Institute (RKI), Berlin, Germany
Gentamycin (10mg/ml)	PAA Laboratories GmbH- VWR International GmbH, Dresden, Germany
Bidest H_2O	RKI, Berlin
L-Glutamine	PAA Laboratories GmbH- VWR International GmbH, Dresden, Germany
Non-essential amino acids (NEAA), 100x	Biochrom GmbH, Berlin, Germany
Sodium hydrogen carbonate (NaHCO3)	PAA Laboratories GmbH- VWR International GmbH, Dresden, Germany
Trypsin-EDTA (1x)	Biochrom GmbH, Berlin, Germany
Tris-HCL	PAA Laboratories GmbH- VWR International GmbH, Dresden, Germany
10X PCR Buffer (2M Tris-HCl+5M KCl)	Invitrogen GmbH (DE), Karlsruhe, Germany
5X RT Buffer	Invitrogen GmbH (DE), Karlsruhe, Germany
100bp Generuler/DNA-Low Mass Ladder	Invitrogen GmbH (DE), Karlsruhe, Germany
H_2O RNase free	Sigma-Aldrich, Munich, Germany
MEM (Minimum essential medium)/Hepes	Invitrogen- Thermo Fisher Scientific GmbH, Schwerte, Germany
DNA Gel –Loading buffer (6X)	Invitrogen GmbH (DE), Karlsruhe, Germany
Phosphate buffered saline (PBS)	Invitrogen- Thermo Fisher Scientific GmbH, Schwerte, Germany
TAE-buffer (50%)	Invitrogen- Thermo Fisher Scientific GmbH, Schwerte, Germany
5 x ABI sequencing buffer	Applied Biosystems, Darmstadt, Germany
BigDye 3.1 buffer	Applied Biosystems, Darmstadt, Germany
Random primers	Invitrogen- Fisher Scientific, Schwerte, Germany
Enzyme	**Source**
Reverse Transcriptase (RT)	Invitrogen GmbH, Karlsruhe, Germany
Platinum Taq DNA Polymerase	Invitrogen GmbH, Karlsruhe, Germany
Ex Taq DNA Polymerase, Hot-Start Version	Takara Bio Europe/Clontech Laboratories, Inc., Saint-Germain-en-Laye, France
RNasin®RNase Inhibitor	Promega, Mannheim, Germany

Receptor destroying enzyme (RDE)	Sigma-Aldrich, Munich, Germany

2.1.3 Media and solutions

Cell culture media	Preparation
MEM/Hepes growth culture medium (10%)	10% FCS (inactivated 30min by 56°C) + 1% L-Glutamine + 1% NEAA+0.5ml Gentamycin/100ml medium + 1% Pyruvate in 500ml medium (MEM/Hepes), pH 7.2.
MEM/Hepes maintenance medium (2%)	2% FCS + 0.5 ml Gentamycin/100 ml Medium + 1% L-Glutamine + 1% NEAA + 1% Pyruvate, in 500ml medium (MEM/Hepes), pH 7.2.
MEM/trypsin-EDTA infection medium	0.2% trypsin-EDTA + 0.5 ml Gentamycin/100 ml Medium + 1% L-Glutamine + 1% NEAA, in 500ml medium (MEM/Hepes), pH 7.2.
Solution	**Preparation**
TAE-buffer, 50x	242g TrizmaBase + 57.1ml CH3COOH (100%) + 100ml EDTA (0.5M) + bidest H_2O, added to be 1L.
TAE-gel running buffer, 1x	50ml TAE-buffer (50x) + bidest H_2O, added to be 1L.
1.5% Agarose gel solution	8g of agarose powder+4mls 50x TAE buffer + 300ml bidest H_2O; dissolved on heat and added up to 400ml.

2.1.4 Kits

Kit	Source
Big Dye Terminator v3.1	Applied Biosystems, Darmstadt, Germany
MSB® Spin PCRapace purification kit	Stratec Molecular GmbH, Birkenfeld, Germany
Invisorb® Spin DNA Extraction kit	Stratec Molecular GmbH, Birkenfeld, Germany
Invitek RTP®DNA/RNA Virus Mini Kit	Stratec Molecular GmbH, Birkenfeld, Germany
UTM™ Viral Transport Media/sample collection kit	Copan Diagnostics, Brescia, Italy

2.1.5 Antisera used for HI test of influenza viruses

Name	Antiserum	Source
Influenza A/H1	A/California/7/09	WHO Influenza Centre, National Institute for Medical Research, London, UK
Influenza A/H3	A/Texas/50/2012	
	A/Switzerland/9715293/13	
Influenza B	B/Brisbane/60/2008	
	B/Massachusetts/2/2012	
	B/Phuket/3073/13	

2.1.6 Oligonucleotides

2.1.6.1 Oligonucleotides for real-time PCR

Oligonucleotides[a]	Polarity	Oligosequence (5'→3')	Gene	nM
IV A/RSV/FCV triplex [228]				
Influenza A virus				
M+25	Forward	AGATGAGTCTTCTAACCGAGGTCG	M	300
M-124sw	Reverse	CTGCAAAGACACTTTCCAGTCTCTG	M	300
M-124 BB	Reverse	CCWGCAAARACATCYTCAAGTYTCTG	M	600
MGB M+64	Probe	6-FAM-TCAGGCCCCCTCAA	M	100
RSV				
RSV L F 2014	Forward	GTGGAACTTCATCCTGAYATAAGATATATT	L	600
RSV L R 2014	Reverse	GTTGCATCTGTAGCRGGAATGGT	L	600
RSV L MGB 2014	Probe	VIC- TTGCAATGATCATAGTTTACC	L	100
FCV				
FCV F54	Forward	CGTTACCGCCACACCCAT	Pol-P	300
FCV R141	Reverse	GAGTTCACGAAAGATTTCAGACCAT	Pol-P	300
FCV TM96	Probe	LC610-ACCCATCATTCTAACACTCCC-GCCAAT	Pol-P	100
HMPV/RV duplex [145]/(J. Reiche, RKI)				
HMPV				
HMPV F S	Forward	GCTCCGTAATYTACATGGTGCA	F	500
HMPV F S1	Forward	GAAGCTCYGTGATTTACATGGTACA	F	500
HMPV F AS	Reverse	GACCCTGCARTCTGACAATACCA	F	500
HMPV F AS1	Reverse	AGTKGATCCTGCATTTTTACAATACCA	F	500
HMPV F TMGB	Probe	6FAM-CCYTGCTGGATAGTAAAA	F	100
HMPV F TMGB1	Probe	6FAM-CCTTGTTGGATAATCAA	F	100
RV				
HRV 375 F1	Forward	GTGKYCYAGCCTGCGTGGC	5´ UTR	300
HRV 586 R1	Reverse	ACGGACACCCAAAGTAGTYGGT	5´ UTR	300
S-HRV 476 –BBQ	Probe	YAK-CCTCCGGCCCCTGAATGYGGCTAA	5´ UTR	100
IV B singleplex [229]				
FluB HA YamVic F432	Forward	ACCCTACARAMTTGGAACYTCAGG	HA	900
FluB HA YamVic R479	Reverse	ACAGCCCAAGCCATTGTTG	HA	600
FluB HA Yam MGB437	Probe	6-FAM-AATCCGMTYTTACTGGTAG	HA	150
FluB HA Vic MGB470	Probe	VIC-ATCCGTTTCCATTGGTAA	HA	100
HAdV singleplex [230]				
P-033	Forward	GAGAAAGGACGCCGCCTTATGGA	DPol	100
P-033	Reverse	CAAACAGTTTCACATTCAACTGACCAGG	DPol	100
P-034	Forward	GGGGACACCGGCTCATGGA	DPol	100
P-034	Reverse	GTTTCGCATTCCACGAGCCAGG	DPol	100
P-035	Forward	AGCCGGATACCGCCTCATGGA	DPol	100
P-035	Reverse	GGTTTCGCACTCCACTAACCAGG	DPol	100
P-039	Forward	GCGCGGACACAGACTCATGGA	DPol	100
P-039	Reverse	TCGCACTCGACGAGCCAGG	DPol	100
P-040	Forward	CTCAACGAGGACACGAACTCATGGA	DPol	100
P-040	Reverse	CAGTTTCACATTCCACCAGCCAGG	DPol	100
MGB033/1– MGB/NFQ	Probe	FAM - AGGTAAGAAACGCATCAAA	DPol	50
MGB033/2– MGB/NFQ	Probe	FAM - AGGTAAGAAGCGCATCAA	DPol	50
HBoV singleplex (B. Biere, RKI)				
Boca F2446	Forward	TACAAAAGAAAAGGGAGTCCAGAAA	NP1	300
Boca R2518	Reverse	TCCTGCTCCTGTGATGAGTTGT	NP1	200

Boca MGB2493	Probe	CCAGTGTCTCTTCCT	NP1	100
IV A (H1/H3) duplex [228]				
Influenza A H1virus				
FluA H1pdm F236	Forward	TGGGAAATCCAGAGTGTGAATCACT	HA	300
FluA H1pdm R318 2014	Reverse	CGTTCCATTGTCTGAACTAGATGTT	HA	300
FluA H1pdm TM292+ 2014	Probe	LC610-CCACAATGTAGGACCATGARCTTGC TGTG	HA	150
Influenza A H3 virus				
Influenza H3 2014				
H3F-162 2014	Forward	GACAGTCCTCATCAGATCCTTGATG	HA	300
H3R-291 2014	Reverse	GGTAACAGTTGCTGTRGGCTTTGC	HA	300
H3S-284 MGB	Probe	VIC-CTCTATTGGGRGACCC	HA	100
IV A (N1/N2) duplex [228]				
Influenza A N1 virus				
FluA N1pdm F1255 2014	Forward	AGACCTTGCTTCTGGGTTGAAC	NA	300
FluA N1pdm R1334	Reverse	AAGGATATGCTGCTCCCRCTAGT	NA	300
FluA N1pdm TM1310 2014	Probe	6-FAM-CAGATTGTGTTCTCTTYGGGT-CGCCCT	NA	100
Influenza A N2 virus				
FluA N2 F769 2014	Forward	TGTTACTAAAATACTATTCATTGAGGAGGG	NA	300
FluA N2 R892	Reverse	GCAGACACATCTGACACCAGGATAT	NA	300
FluA N2 TM804	Probe	LC610-TCGTTCATACTAGCACATTGTCAGG-AAGTGC	NA	100
RSV (A/B) duplex [66]				
RSVA				
P-RSVA-G409	Forward	AAGACCAAAAACACAACAACAA	G	600
P-RSVA-G586Neu	Reverse	TTGGTATTCTCTTGCAGATGG	G	300
S-RSVA-G-556-BBQ	Probe	YAK-TTGGATTGTTGCTGCATATGCTGCT	G	100
RSV B				
P-RSVB-G155	Forward	CAATGATAATCTCAACCTCTCTCA	G	300
P-RSVB-G303	Reverse	GGTGAGACTTGAGTAAGGTAAGTG	G	300
S-RSVB-G-201-BBQ	Probe	6FAM-CATCTCTGCCAATCACAAAGTTACA-CTAACAAC	G	150
HPIV quadruplex (J. Reiche, E. Obodai, RKI; adopted from [231])				
HPIV1				
HPIV1_RKI_TM_s	Forward	TTGGTGATGCAATATATGC	HN	300
HPIV1_RKI_TM_as	Reverse	RTA ACC TAA TTG TAA AAC CTG	HN	300
HPIV1_RKI_TM/MGB_Sonde	Probe	FAM CACTCAAGGATGTGCAGATATAGG	HN	150
HPIV2				
HPIV2_TM_s	Forward	CCATTTACCTAAGTGATGGAA	HN	300
HPIV2_RKI_TM_as	Reverse	ACA ACCTCCTGGTATAGC	HN	300
HPIV2_TM/MGB_Sonde	Probe	VIC-AATCGCAAAAGCTGTTCAGTCAC	HN	150
HPIV3				
HPIV3_TM_s	Forward	GRAGCATTGTRTCATCTGTC	HN	300
HPIV3_TM_as	Reverse	TAGTGTGTAATRCAGCTTGT	HN	300
HPIV3_TM_Sonde – BBQ	Probe	TEX-ACCCAGTCATAACTTACTCAAC-AGCAAC	HN	150
HPIV4				
HPIV4_RKI_TM_s	Forward	ATCAAGACAATACAATTACACTTGA	P	300
HPIV4_RKI_TM_as	Reverse	CTGTTATTTTAAGTGCATCTATAC	P	300
HPIV4_RKI_TM_Sonde – BBQ	Probe	CY5-RTTGGTTCCAGAYAAWATGGGTC-TTGCTA	P	150
HCoV quadruplex (B. Biere, RKI)				

HCoV-NL63				
NL-63 F	Forward	AACGTGTTGATTTGCCTCCTAA	N	300
NL-63 R	Reverse	GTTTGCGATTACCAAGACTGG	N	300
NL-63 TMGB	Probe	FAM-CTTATGAGGTCCAGTACC	N	100
HCoV-229E				
229E F	Forward	TACCACACTTCAATCAAAAGCTCC	N	300
229E R 2014.2	Reverse	GCGACTCTGMGACCTYGACT	N	300
229E TMGB	Probe	VIC-CACGGGAGTCAGGTTCT	N	100
HCoV-OC43				
TIB OC43 F	Forward	CGATGAGGCTATTCCGACTAGGT	N	300
TIB OC43 R	Reverse	CCTTCCTGAGCCTTCAATATAGTAACC	N	300
TIB OC43 TM	Probe	TCCGCCTGGCACGGTACTCCCT	N	100
HCoV-HKU1				
HKU1 Dare RKI F	Forward	CTTGCGAATGAATGTGCWCAAG	N	300
HKU1 R	Reverse	TTGCATCACCACTGCTAGTACCAC	N	300
HKU1 Dare TM BHQ-3	Probe	CY5-GTGTGGCGGTTGCTATTATGTTA-AGCCTG	N	100

[a] All oligonucleotides were purchased from Tib Molbiol GmbH (Berlin, Germany) and Metabion (Martinsried, Germany). Wobbles: K = A/G; M = G; R = A/G; X = any amino acid base; W = G; Y = C/U.

Abbreviations: nM, nanomoles, equals final concentration in PCR reaction.

Flourescence dyes: FAM = 6-Carboxyfluorescein; VIC = VIC; TEX = Texas red; CY5 = Cyanine 5; YAK = Yakima yellow; MGB = Minor groove binder; NFQ = Non fluorescence quencher.

Viral protein genes: M = Matrix; L = Large polymerase; P-Pol = Polyprotein; F = Fusion protein; UTR = Untranslated region; DPol = DNA polymerase; NP1 = Nonstructural protein; HA = Hemagglutinin; NA = Neuraminidase; G = Glycoprotein; HN = Hemaglutinin-neuraminidase; P = Phosphoprotein; N = Nucleocapsid.

Respiratory viruses: IV = influenza virus; RSV = Respiratory syncytial virus; FCV = Feline calicivirus; HMPV = Human metapneumovirus; RV = human rhinovirus; HAdV = Human adenovirus; HBoV = Human bocavirus. HPIV = Parainfluenza virus; HCoV = Human coronavirus.

2.1.6.2 Probes for HAdV - Fluorescence curve melting analysis

Probes[a]	Polarity	Oligosequence (5'→3')	Gene	nM
FCMA HAdV [230]				
LC033/A	Probe	CCGTACTTTTTGATGCGTTTC-FL	DPol	150
LC033/B	Probe	LC RED 640 TACCTTGCGACTCCATAA AAAAACAAGTTTTCCGCCAT-FL	DPol	150
LC033/C	Probe	LC-RED-640-TTTTTTGATGCGTTTCTTACCTTGGT GTGAGCTCTGGCCGTTCGG-FL	DPol	150
LC033/D	Probe	LC-RED-640-GTCAAAAACCAGGTTTCCCC GTAAGAAGCGCATCAAAAAGAACG-FL	DPol	150
LC033/E	Probe	LC-RED-640-GGGAAAACTGGTTTTTGACCCCGA CAAAAAGAACGGCGGAAAACTGGTTT-FL	DPol	150
LC033/F	Probe	LC-RED-640-GATCCCAATCAGCCCGACC CGTGTTTTTTGATGCG-FL	DPol	150
		LC-RED-640-TTCTTACCTCGGGTTTCCATGAG		

[a] Probes were purchased from Tib Molbiol GmbH (Berlin, Germany). Abbreviations: nM, nanomoles, equals final concentration in PCR reaction. Hybridization probes: FL = fluorescence transmiter dye; LC = Flourescence acceptor dye; DPol = DNA polymerase

2.1.6.3 Oligonucleotides for conventional-PCR and sequencing

Oligonucleotide[a]	Polarity	Oligosequence (5'→3')	Gene	nM
IV A (M. Wedde, RKI)				
Influenza A(H1N1)pdm09 virus				
FluA-H1 pdm09 virus				
H1-09 Seq 2015 F1	Forward	AGCAAAAGCAGGGGAAAAYAAA	HA	750
H1-09 Seq 2015 R1362	Reverse	CCAACAGTTCGGCATTGTAAG	HA	750
H1-09 Seq 2015 F1163	Forward	ATATGCAGCCGACCTGAAGAG	HA	750
H1-09 Seq 2015 R1770	Reverse	ACAAGGGTGTTTTTCTCATGCTTCT	HA	750
H1-09 Seq 2015 F587	Forward	TAAAGGGAAAGAAGTCCTCGTG	HA	750
H1-09 Seq 2015 R721	Reverse	TCCGGCCTTGAACTTCTTGCT	HA	750
FluA-N1 pdm09 virus				
FluSw N1 F22	Forward	TGAATCCAAACCAAAAGATAATAACCA	NA	750
FluSw N1 R877	Reverse	CTAGAATCAGGATAACAGGAGCATTCC	NA	750
FluSw N1 F726 2011	Forward	GGTTCTTGCTTTACYRTAATGACCG	NA	750
FluSw N1 R1452	Reverse	AACAAGGAGTTTTTTGAACAAATTACTTG	NA	750
Influenza A(H3N2) virus				
FluA-H3 virus				
H3 Seq 2015 F1a	Forward	AGCAAAAGCAGGGGATAATTC	HA	750
H3 Seq 2015 R1350	Reverse	CGTTGTATGACCAGAGATCTATTTTWGT	HA	750
H3 Seq 2015 F1140	Forward	CATCAAATTCTGAGGGAAGAGG	HA	750
H3 Seq 2015 R1761	Reverse	GTAGAAACAAGGGTGTTTTTAATTAATG	HA	750
H3 Seq 2015 F568	Forward	TGAACGTGACTATGCCAAACAA	HA	750
H3 Seq 2015 R726a	Reverse	TATTYGGGATTACAGCTTGTTGG	HA	750
FluA-N2 virus				
FluA N2 F11	Forward	AGTGAAGATGAATCCAAATCAAAAGA	NA	750
FluA N2 R807 2011	Reverse	GAACGATTTTCCCCTCCTCRA	NA	750
FluA N2 F692 2010	Forward	CCAGGAGTCRGAATGCGTYTG	NA	750
FluA N2 R1430 2011	Reverse	AGCTTATATAGGCATGAGATTGAKRTYC	NA	750
IV B (A. Heider, RKI)				
Influenza B/Victoria virus				
Flu B HA virus				
B Seq F7 2014	Forward	AGCAKWGCATTTTCTAATATCC	HA	750
BVic Seq R1021a 2014	Reverse	CATGTTCCCCTGTGTAGTAAG	HA	750
BVic Seq F881 2014	Forward	TATTGCCTCAAAAGGTGTG	HA	750
BVic Seq R1861a 2014	Reverse	TAACGTTTCTTTGTAATGRCA	HA	750
BVic Seq F406a 2014	Forward	CTTCTCMGAGGMTACGAA	HA	750
BVic Seq R638 2014	Reverse	TGGTCTTCTCCTTCTGTACAAA	HA	750
Flu B NA virus				
FluB NA F1	Forward	AGCAGAAGCAGAGCATMTTC	NA	750
FluB NA R1153	Reverse	CGAGAGTACCACCTTCCAA	NA	750
FluB NA F1025 2012	Forward	CACCCCCAGACCARABGA	NA	750
FluB NA R1557	Reverse	AGTAGTAACAAGAGCATTTTTCAGA	NA	750
FluB NA F500	Forward	CAGAAACAAGCTGAGGCA	NA	750
FluB NA R580	Reverse	GCTGCCATGTGGAAAAT	NA	750
RSV A [66]				
RSVA-G-F	Forward	AGTGTTCAACTTTGTACCCTGC	G	250
RSV-F-R	Reverse	CTGCACTGCATGTTGATTGAT	F	250
RSVA-G-606-F[b]	Forward	AACCACCACCAAGCCCACAA	G	250
RSV-F-22-R[b]	Reverse	CAACTCCATTGTTATTTGCC	F	250
RSV B [66]				
RSVB-G-524-F	Forward	TTGTTCCCTGTAGTATATGTG	G	250

RSV-F-55-R	Reverse	AGTTAGGAAGATTGCACTTGA	F	250
RSVB-G-603-F[b]	Forward	AAAACCAACCATCAAACCCAC	G	250
RSV-F-22-R[b]	Reverse	CAACTCCATTGTTATTTGCC	F	250
HMPV [145]				
HMPV-3637-F	Forward	GTYAGCTTCAGTCAATTCAACAGAAG	F	250
HMPV-4192-R1	Reverse	CAGTGCAACCATACTGATRGGATG	F	250
HMPV-4192-R2	Reverse	TAGTGCAACCATACTGATRGGGTG	F	250
HMPV-3637-F[b]	Forward	GTYAGCTTCAGTCAATTCAACAGAAG	F	250
HMPV-4164-R[b]	Reverse	CCTGTGCTRACTTTGCATGGG	F	250
RV (J. Reiche, RKI)				
HRV-Seq-F2-out	Forward	CGGCCCCTGAATGCGGCTAA	VP4/2	600
HRV-Seq-9565-R	Reverse	GCATCIGGYARYTTCCACCACCANCC	VP4/2	900
HRV-Seq-9895-F[b]	Forward	GGGACCAACTACTTTGGGTGTCCGTGT	VP4/2	300
HRV-Seq-9565-R[b]	Reverse	GCATCIGGYARYTTCCACCACCANCC	VP4/2	300
HAdV (B. Biere, RKI, adopted from [232])				
HAdV A				
HAdV Fiber A Xu F	Forward	GCTGAAGAAMCWGAAGAAAATGA	Fiber	500
HAdV Fiber A Xu R	Reverse	CRTTTGGTCTAGGGTAAGCAC	Fiber	500
HAdV B				
HAdV Fiber B Xu F	Forward	TSTACCCYTATGAAGATGAAAGC	Fiber	500
HAdV Fiber B Xu R	Reverse	GGATAAGCTGTAGTRCTKGGCAT	Fiber	500
HAdV HVR PCR B F1	Forward	GCATACATGCACATCGCCG	Hexon	500
HAdV HVR PCR B R1	Reverse	AGAACGGTGTACGCAGGTAGAC	Hexon	500
HAdV HVR PCR B F2[b]	Forward	GACAGGATGCTTCGGRGTACC	Hexon	500
HAdV HVR PCR B R2[b]	Reverse	GCTGATGCACTCTGACCACG	Hexon	500
HAdV HVR Seq B1 F571[c]	Forward	CCAGARCCTCARGTKGGA	Hexon	500
HAdVHVR Seq B1 F1043[c]	Forward	X TGAATGCDGTGGTTG ACTT XX	Hexon	500
HAdVHVR SeqB1 R1116[c]	Reverse	XXXX GTCHCCCAG AGARTCAAGC X	Hexon	500
HAdV HVR Seq B1 R679[c]	Reverse	XXX ACCCRTAGC AKGGYTTCAT	Hexon	500
HAdV C				
HAdV Fiber C Xu F	Forward	TATTCAGCATCACCTCCTTTCC	Fiber	500
HAdV Fiber C Xu R	Reverse	AAGCTATGTGGTGGTGGGGC	Fiber	500
HAdV HVR PCR C F1	Forward	ATGATGCCGCAGTGGTCTTAC	Hexon	500
HAdV HVR PCR C R1	Reverse	ATTAAAGGACTGGTCGTTGGTGTC	Hexon	500
HAdV HVR PCR C F2[b]	Forward	ACGACGTRACCACAGACCG	Hexon	500
HAdV HVR PCR C R2[b]	Reverse	GCCACCACTCGCTTGTTCAT	Hexon	500
HAdV HVR Seq C F652[c]	Forward	GGMGAATCTCAGTGGWAYGAA	Hexon	500
HAdV HVR Seq C F1183[c]	Forward	TAYTTTTCYATGTGGAAKCAGGC	Hexon	500
HAdV HVR Seq C R1148[c]	Reverse	XXX TGRTAKGAM AGCTCTGTGTTTCTG	Hexon	500
HAdV HVR Seq C R744[c]	Reverse	XX ATANGAWCC RTARCATGGTTTCAT	Hexon	500
HAdV D				
HAdV Fiber D Xu F	Forward	GATGTCAAATTCCTGGTCCAC	Fiber	500
HAdV Fiber D Xu R	Reverse	TACCCGTGCTGGTGTAAAAATC	Fiber	500
HAdV HVR PCR D F1	Forward	CCCTCGATGATGCCGC	Hexon	500
HAdV HVR PCR D R1	Reverse	ACTGGTCGTTGGTGTCGTTG	Hexon	500
HAdV HVR PCR D F2[b]	Forward	CGCCTCGGAGTACCTGAGCC	Hexon	500
HAdV HVR PCR D R2[b]	Reverse	GGATGTGGAAGGGCACGTA	Hexon	500
HAdV HVR Seq D F333[c]	Forward	CAGCTTCAAACCCTACTCGG	Hexon	500
HAdV HVR Seq D F1023[c]	Forward	GGTCGACTTGCAAGACAGAAA	Hexon	500
HAdV HVR Seq D R355[c]	Reverse	TGCCCGAGTAGGGTTTGAA	Hexon	500
HAdV HVR Seq D R998[c]	Reverse	CCAGCCAGCACACCCAT	Hexon	500
HAdV E				
HAdV Fiber E Xu F	Forward	TCCCTACGATGCAGACAACG	Fiber	500

HAdV Fiber E Xu R	Reverse	AGTGCCATCTATGCTATCTCC	Fiber	500
HAdV F				
HAdV Fiber F Xu F	Forward	ACTTAATGCTGACACGGGCAC	Fiber	500
HAdV Fiber F Xu R	Reverse	TAATGTTTGTGTTACTCCGCTC	Fiber	500

[a] All oligonucleotides were purchased from Tib Molbiol GmbH (Berlin, Germany) and Metabion (Martinsried, Germany). [b] Primers for semi/nested PCR reaction. [c] Primers for sequencing only.

Wobbles: K = A/G; M = G; N = C/U; R = A/G; S = A/ C/G/U; X = any amino acid base; W = G; Y = C/U.

Abbreviations: nM, nanomoles, equals final concentration in PCR reaction.

Viral protein genes: HA = Hemagglutinin; NA = Neuraminidase; G = Glycoprotein; F = Fusion protein; VP4/2 = Capsid protein.

Respiratory viruses: IV = influenza virus; RSV = Respiratory syncytial virus; HMPV = Human metapneumovirus; RV = human rhinovirus; HAdV = Human adenovirus

2.1.7 Software and databank

Software	Source
DNASTAR Lasergene 10 Core Suite,	DNASTAR Inc., Madison, USA
BioEdit Sequence Alignment Editor Version 7.2.5.	Ibis Biosciences, Carlsbad, CA
MEGA (Molecular Evolutionary Genetic Analysis) versions 5.2, 6.06, 7.2.5	www.megasoftware.net/
GISAID (global initiative on sharing all influenza data)	database, platform.gisaid.org/
NCBI databank	http://www.ncbi.nlm.nih.gov
The SPSS program version 20	SPSS Inc. Chicago, USA
NetNGlyc 1.0 server and NetOGlyc 3.1 server	www.cbs.dtu.dk/services/NetNGlyc/
SNAP (Synonymous/Nonsynonymous Analysis Program) database	http://www.hiv.lanl.gov/content/sequence/SNAP.html
LightCycler 480II version 1.5.1.62 SP1	Roche, www.roche-applied-science.com
Microsoft office 2010 (word, excel, powerpoint)	2010 Microsoft Corporation
Corel photo paint x6, version 16.2.0.998	Corel, Ottawa, Canada
BioDocAnalyze version 2.86.3.15,	Biometera 10, Analytik Jena company, Germany

2.2 Methods

2.2.1 Patient enrollment and sampling

Children below five years of age with ALRI were enrolled into a cross-sectional hospital based survey, from February to November 2006 and January 2013 to December 2014. The children were recruited from two hospitals; the KBTH and the PMLCH. The study hospitals are located in Accra, Ghana. The KBTH is a tertiary and national referral health care facility [233]. The Child Health Department of KBTH has an average out-patient attendance of 36,000 per year. It also has special facilities including three medical wards, a surgical ward, a neonatal intensive care unit and specialty clinics [233]. The PMLCH is a community hospital which offers primary health care to children less than 15 years within the Greater Accra metropolis and its immediate environs [234]. The hospital has an average out-patient attendance of 73,000 per year. The PMLCH also has a 3-storey theater and recovery ward, an intensive care unit and other support units such as Nutrition Rehabilitation Centre, X-ray and Laboratory units.

Patients who met the eligibility criteria for recruitment into the study were enroll on a daily basis, and participation was voluntarily. Prior to enrollment, parents or caregivers gave written informed consent after careful explanation of procedures in English language or local dialect. A standardized questionnaire form was used to record the history of illness as well as the presenting clinical features of the patients. Eligible patients were defined as follows:
a) Children <2 months having breathing rate of greater than or equal to 60 breaths/minute,
b) Children 2-11 months having breathing rate of greater than or equal to 50 breaths/minute,
c) Children 12- 59 months having breathing rate of greater than or equal to 40 breaths/minute, in addition to either a cough or nasal discharge or fever. Known asthmatics and children with abnormal cardiovascular systems were excluded from the study. The study was approved by the University of Ghana Medical School Ethical and Protocol Review Committee, College of Health Sciences, University of Ghana.

Two types of clinical specimens were obtained; nasopharyngeal aspirates [235] and nasal swabs (NS). NPA were collected by aspiration through a sterile single-use catheter and washed down with 2ml buffered saline solution into a sterile disposable test tube, while NS were taken using a sterile flexible flocked swab and placed in a 1ml UTM. Specimens were placed on ice and transported within few hours of collection to the Department of Microbiology, University of Ghana Medical School, where they were stored at -80^{o}C until

shipment to the National Influenza Center at the Robert Koch Institute Berlin, Germany, for virological investigations. Upon arrival in the laboratory, 3mls of sterile minimal essential medium containing 100U/ml penicillin-streptomycin was added to each respiratory sample. Samples were vortexed and further prepared into three aliquots as follows: 1ml aliquot for PCR reaction, 1ml filtered aliquot for cell culture virus isolation, and 1ml aliquot as backup storage.

2.2.2 Cell culture and virus isolation

2.2.2.1 Cell culture conditions

MDCK-SIAT cell lines were grown using growth medium (see 2.1.3.1) at 37°C. Culturing of the cell monolayer was performed in 75cm tissue culture flasks. The growth medium was discarded and replaced with maintenance medium (see 2.1.3.1) after every two days. Confluent cells (between 80-100%) were washed with PBS, detached using 5ml 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, and 1:6 for preparation of tubes for virus isolation.

2.2.2.2 Influenza virus isolation

MDCK-SIAT cells were inoculated with 200µl of filtered sterile specimens influenza virus positive samples (as determined by real time PCR) in culture tubes with 2mls of infection medium (see 2.1.3.1). Culture tubes were incubated at 33^{o}C and examined daily for virus growth by a cytopathic effect (CPE)/rounding of cells. The medium was changed every two days with maintenance medium (see 2.1.3.1) if no CPE was observed, and passage into new tubes after six days. Following 14 days of culture, cells with no CPE were discarded as negatives. CPE formation generally occurred after three or four days post-infection. Virus isolates were harvested from culture tubes for further identification.

2.2.3 Hemagglutination assay for influenza virus titer determination

The hemagglutination (HA) assay was used to determine the titer of the influenza virus isolates obtained from all culture (see 2.2.2.2). Based on their ability to attach to receptors present on the surface of red blood cells (RBCs), a virus may agglutinate the RBCs, thus

preventing them from settling down in the plate. In the absence of hemagglutination (as in negative control wells), the RBCs form a compact lump on the bottom of the wells. The influenza virus suspensions were serially diluted in twofold (2^{-1} to 2^{-7}) in a 50µl final volume in a V-bottom microtiter plate. The dilutions were mixed with an equal volume of turkey and guinea pig RBCs (0.5%, vol/vol), and incubated at room temperature for 30min (turkey RBCs) or 60min (guinea pig RBCs). The RBCs type to which the virus reacted better and showed the highest HA titer was preferred and used in subsequent tests. The endpoint dilution was considered one hemagglutination unit (1HAU) and the number of HAUs/50µl dilution (virus HA titer) was determined by a simple number or reciprocal of the highest dilution factor that produced a positive reading.

2.2.4 Hemagglutination Inhibition assay for influenza virus antigenic characterization

The hemagglutination inhibition (HI) test was performed using a panel of specific post-infection ferret sera, for all influenza virus isolates that did agglutinate RBCs (see 2.2.3). The presence of antibodies to a particular influenza virus subtype or variant will prevent attachment of the virus to RBC, thereby inhibiting hemagglutination formation. The ferret immune sera which were used included antiserum against the vaccine strain A/California/7/09 for analysis of A(H1N1)pdm09) viruses, antisera against the vaccine strains A/Texas/50/2012 and A/Switzerland/9715293/13 for A(H3N2) virus analysis and antisera against B/Brisbane/ 60/2008, B/Massachusetts/2/2012 and B/Phuket/3073/13 vaccine strains for analysis of influenza B viruses. Prior to testing, each post-infection ferret antiserum was treated with receptor destroying enzyme to inactivate non-specific inhibitors achieving a final serum dilution of 1:20. A twofold serial dilution of the sera was prepared in V-bottom microtiter plates and an amount of virus equivalent to 4HAunits/25µl was added to every well, except for the serum control wells and incubated at room temperature for 30min. Turkey or guinea pig RBCs were added, plates agitated briefly and were then allowed to stand at room temperature for another 30 or 60min, respectively. Observation of movement of RBCs at the button when the plate was tilted helped to clarify the end point determination. The reciprocal of the highest dilution of serum that prevents hemagglutination corresponded to the HI titer of the serum.

2.2.5 Validation of the real-time multiplex PCR for the detection of HPIV

Screening of respiratory samples for HPIV was performed with a new validated, specific and sensitive two-step real-time multiplex RT-PCR assay. Therefore annealing temperature, $MgCl_2$ concentration, and primer/probe concentrations were first evaluated for each of the single assays to reach optimal reaction conditions detecting HPIV1, HPIV2, HPIV3, and HPIV4. The specificity of each single assay was evaluated with nucleic acids of circulating HPIV types (1, 2, 3, 4a and 4b) and other respiratory pathogens including influenza virus types A and B, human HMPV RSVA and B, HAdV 2–4, RV 1B and 37, human echovirus 6, 9, 11 and 19, human coxsackievirus A4 and B3, human rhinovirus 1B and 37, *Streptococcus pneumoniae* type 14 and 23, *Staphylococcus aureus* type 4 and 5 and *Chlamydia pneumoniae*. All of the nucleic acids had been previously tested positive with specific PCR assays. All assays were 100% specific for HPIV; no amplification was obtained with the other viral or bacterial respiratory pathogens tested. The sensitivity of each assay was determined by amplification of 10-fold serial dilutions of plasmids containing the respective PCR target sequence in Lambda (λ) DNA (1 ng/µl). Each assay revealed a linear detection range from 10^6 to 10^1 genome equivalents per reaction. A strong correlation coefficient $R^2 > 0.996$ over the 5-log range was also achieved for each assay. Additionally the assays were multiplexed in a single tube and the reaction mix was examined for PCR efficiency. The multiplex mix correspondingly achieved a linear amplification with a high standard curve correlation of $R^2 \geq 0.998$ for each plasmid target. The intra- and inter-assay reproducibility was assessed using 10-fold serial dilutions of plasmids in triplicate in a single run (for intra-assay), and in duplicate on three different days (for inter-assay). The intra-assay variation ranged from 0.30-3.26% and the inter-assay variation was from 0.90-2.81%. The 95% detection probability (probit analysis) was found to be 14.1, 28.0, 64.8 and 28.3 genome equivalents per reaction for HPIV1, HPIV2, HPIV3 and HPIV4, respectively. The multiplex assay was successfully validated with 30 clinical specimens previously shown to be positive for HPIV by an alternative HPIV-PCR.

2.2.6 Extraction of nucleic acids from specimen and virus suspensions

Viral RNA and DNA were extracted from 400ul of prepared samples and/or 200µl culture supernatant (adjusted to 400µl with MEM) using RTP®DNA/RNA Virus Mini Kit according to the manufactures' instructions. The total elution volume was 60µl. For an internal

extraction control, samples were spiked with 20µl Feline calicivirus to yield approximately 50 genome equivalents per PCR reaction.

2.2.7 Reverse transcription of viral RNA

Synthesis of cDNA was performed with 25µl of RNA in a 40µl mixture containing a 250nM of random hexamer primers, 200µM of each deoxynucleoside triphosphate (dNUTPs), 5mM dithiothreitol, 20U RNasin, 100U Moloney murine leukemia virus reverse transcriptase and first-strand buffer containing 250mM Tris-HCl (pH 8.3), 37.5mM KCl, and 15mM $MgCl_2$. The reaction was carried out for 5min at 42°C, followed by 30min at 37°C, and finally for 5min at 94°C in the Biometra T300 themocycler.

2.2.8 Real-time PCR amplification and detection of respiratory viruses

Different real-time PCR assays were used to analyze the viral cDNA/DNA material for the presence of the following pathogens: Influenza A and B viruses, RSV group A and B, HMPV, RV, HAdV, HBoV, HPIV1-4, and HCoV-OC43, -229E, NL63, and HKU1. First, samples were screened for influenza A virus and RSV with the generic triplex PCR assay (IVA/RSV/FCV). This assay includes the extraction and amplification control FCV. Using specific subtyping real-time PCR assays, influenza A virus-positive samples were differentiated into subtypes A(H1N1)pdm09 and A(H3N2) viruses, and RSV-positive samples were genotyped into RSV group A and B. Afterwards all samples were analyzed for the remaining pathogens. The real-time PCR assays and thermal cycler conditions used were as listed in Table 1 and 2. All assays have 100% specificity; no cross-reactivity with non-targeted pathogens or human genomic DNA. The PCR efficiency for each assay approached 100%, with a high standard curve correlation, and a 95% detection probability (limit of detection using probit analysis) as shown in Table 3.

Table 1: Real-time PCR basic reaction mix

	Volume (µl) by real-time PCR assay									
Reagent	**IV A/ RSV/FCV**	**HMPV/ RV**	**IV B**	**HAdV**	**HBoV**	**IV A (H1/H3)**	**IV A (N1/N3)**	**RSV (A/B)**	**HPIV**	**HCoV**
10x PCR-Puffer, Mg-	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	1.50
50mM $MgCl_2$	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	1.20
2.5mM dNUTP	2.0	2.0	2.0	2.0	2.0	2.0	2.0	1.0	2.0	1.20
BSA (50mg/ml)	-	-	-	-	-	-	-	-	-	0.60
Taq Polymerase (5U/µl)	0.1	0.2	0.1	0.1	0.1	0.1	0.1	0.2	0.2	0.2
Template-DNA	3.0	3.0	3.0	5.0	3.0	3.0	3.0	3.0	5.0	5.0
RNase-free H_2O	ad 25	ad 25	ad 25	ad 25	ad 25	ad 25	ad 25	ad 25	ad 25	ad 15

Primer and probes were specifically added to the basic PCR reaction mix as indicated in section 2.1.7.1.

Abbreviations: IV = influenza virus; RSV = Respiratory syncytial virus; FCV = Feline calicivirus; HMPV = Human metapneumovirus; RV = Human rhinovirus; HAdV = Human adenovirus; HBoV = Human Bocavirus; HPIV = Human parainfluenza virus; HCoV = Human coronavirus. ad = added up to the required final volume.

Table 2: Real-time PCR amplification protocol

Programme	**Time (s)**	**Temp (°C)**	**Number of cycles**
Denaturing	300	95	1
Denaturing Annealing/Elongation	15 30	95 60	45
Cooling	∞	4	1

Table 3: Real-time PCR assay detection limits

Assay Name	Genome copies per reaction, range (95% detection probability)
IV A/RSV/FCV	7.8-12.8
HMPV/RV	10.1-61.4
IV B	18.7-36.7
HAdV	10.6-99.5
HBoV	12.0
IV A (H1/H3)	28.8-51.3
IV A (N1/N3)	11.1-18.2
RSV (A/B)	< 10.0
HPIV	14.1- 64.8
HCoV	64.0-203.9

Abbreviations: IV = influenza virus; RSV = Respiratory syncytial virus; FCV = Feline calicivirus; HMPV = Human metapneumovirus; RV = Human rhinovirus; HAdV = Human adenovirus; HBoV = Human Bocavirus; HPIV = Human parainfluenza virus; HCoV = Human coronavirus.

2.2.9 Adenovirus typing by fluorescence melting curve analysis

Fluorescence melting curve analysis (FMCA) was used for the detection of HAdV species based on melting temperature generated by thermal denaturation of a probe-target hybrid. Six pairs of hybridization probes, each specific for a single adenovirus species [39] were used (see 2.1.7.2). The sequences of serotypes of a particular HAdV species are highly homologous but differ from those of the other species. Consequently a hybridization probe pair that is specific for one species has mismatches to the others, thus giving different melting temperatures. The melting temperatures are characteristic for the target sequence/probe-pair combination, making them highly reproducible. Analysis of an amplicon with the different probe pairs will therefore give a distinctive melting pattern of one perfect match and five non-perfect matches, from which the species can be easily deduced [230]. First, amplification of HAdV was performed by a specific real-time PCR assay as described (Table 1). At the end of this reaction, HAdV positive PCR products were harvested from their respective well and used for serotyping by FCMA. The FCMA assay was performed in a 10µl melting mixture using 1-5µl of PCR product depending on the ct-value [230]. The melting mixture contained 1x PCR buffer, 5mM $MgCl_2$, and 150nM of two probes of each HAdV type, in six separate reactions per sample. HAdV positive controls (HAdV18, HAdV3, HAdV2, HAdV19, HAdV4 and HAdV41) for species A-F, respectively, were included in each run. The PCR-products were denatured for 30s at 95°C, cooled for 5s at 40°C (with a maximum ramping rate of 20°C/s)

and continuously reheated to 85°C at a ramping rate of 0.2°C/s, during which the fluorescence data were acquired. Finally the samples were cooled to 37°C. All reactions were performed and the fluorescence data analyzed by the Light Cycler 480II.

2.2.10 Conventional PCR for molecular analysis of viral pathogens

Conventional PCR was used for the amplification of specific genes of the viral pathogens influenza A and B viruses, RSV group A and B viruses, HMPV, HAdV, and RV. Therefore external and/or (semi-) nested reactions were carried out as described in Table 4 and 5. Of the PCR positive sample sequencing was performed as described in 2.2.12.

Table 4: Conventional PCR basic reaction mix

	Volume (µl) by specific PCR assay						
Reagent	**IV A[a]**	**IV B[a]**	**RSV A**	**RSV B**	**HMPV**	**RV**	**HAdV**
External PCR							
10x PCR-Buffer, Mg-	5.0	5.0	5.0	5.0	5.0	5.0	-
10x Ex Tag Buffer, Mg+	-	-	-	-	-	-	2.5
50mM MgCl2	2.0	2.0	2.0	2.0	3.0	2.0	-
2.5mM dNTP	4.0	4.0	2.0	2.0	2.0	2.0	2.0
Taq Polymerase (5U/µl)	0.2	0.2	0.1	0.1	0.2	0.2	-
Ex Taq Polymerase (5U/µl)	-	-	-	-	-	-	0.13
Template (cDNA)	5.0	5.0	5.0	5.0	5.0	4.0	2.0
RNase-free H_2O	ad 50	ad 50	ad 50	ad 50	ad 50	ad 50	ad 25
Semi/nested PCR							
10x PCR-Puffer, Mg-			5.0	5.0	5.0	5.0	2.5
10x Ex Tag Buffer, Mg+			-	-	-	-	2.5
50mM MgCl2			2.0	2.0	3.0	2.0	
2.5mM dNTP			2.0	2.0	2.0	2.0	2.0
Taq Polymerase (5U/µl)			0.1	0.1	0.2	0.2	-
Ex Taq Polymerase (5U/µl)			-	-	-	-	0.13
Template (cDNA)			2.0	2.0	5.0	4.0	1.0
RNase-free H_2O			ad 50	ad 50	ad 50	ad 50	ad 25

[a] There exist no nested PCR for IV A and B.

Primers were specifically added to the basic PCR reaction mix as indicated in section 2.1.7.3.

Abbreviations: IV = influenza virus; RSV = Respiratory syncytial virus; HMPV = Human metapneumovirus; RV = human rhinovirus; HAdV = Human adenovirus : ad = added up to the required final volume.

Table 5: Conventional PCR amplification protocol

Assay	Denaturation		Number of cycles	Denaturation Annealing Elongation		Extension		Cool 4°C
	Time (min)	Temp (°C)		Time (s)	Temp (°C)	Time (min)	Temp (°C)	Time
External PCR								
FluA[a]	5	95	45	30 30 180	94 60 72	5	72	∞
FluB[a]	5	95	45	30 30 180	94 54 72	5	72	∞
RSV A	5	94	40	30 30 45	94 58 72	10	72	∞
RSV B	5	94	40	30 45 60	94 53 72	10	72	∞
HMPV	5	94	40	30 30 45	94 60 72	10	72	∞
RV	5	94	40	30 30 60	94 65 72	10	72	∞
HAdV	2	98	40	15 30 180	98 60 72	10	72	∞
Semi/nested PCR								
RSV A	5	94	40	30 30 30	94 55 72	10	72	∞
RSV B	5	94	40	30 45 45	94 53 72	10	72	∞
HMPV	5	94	40	30 30 45	94 60 72	10	72	∞
RV	5	94	40	30 30 60	94 60 72	10	72	∞
HAdV	2	98	40	15 30 180	98 60 72	10	72	∞

[a] There exist no nested PCR for IV A and B.

2.2.11 Agarose gel electrophoresis of nucleic acids

The amplified PCR products (see 2.2.10) were analyzed by a 1.5% agarose gel electrophoresis. Samples were diluted in 1.5µl of 6x DNA loading buffer, and 5µl were applied to the gel. Running of gels in 1x TAE buffer was done at 60-90V. The amplicons were visualized under UV light and analyzed using the BioDocAnalyzer instrument and software.

2.2.12 PCR product purification and sequencing reaction

The PCR products were purified either directly with MSB®Spin PCRapace purification kit when only single bands were read. For multiple bands, the expected band size was cut from the agarose gel and purified with JETquick spin column technique according to the manufacturer's instructions. Purified PCR products were quantified photometrically by nanodrop measurements or by mass measurement using the BioDocAnalyzer software. By using the ABI PRISM® Big Dye® Terminator v3.1 Cycle Sequencing kit (Applied Biosystems), separate cycle sequencing reactions for each virus gene were set up in both the forward and the reverse directions with the primer pairs used in the external or semi-/nested PCR. The sequencing reaction and cycling conditions were as follows:

Table 6: Sequencing reaction (10µl)

Reagent	Volume (µl)
Template (10-20ng)	2 (1-4)
Primer (10µM)	0.5
BigDye 3.1	1.0
5x ABI Buffer	1.5
HPLC-Grade H_2O	ad 10

Table 7: Cycle sequencing conditions

Programme	Time (s)	Temp (°C)	Number of cycles
Denaturing	120	96	1
Denaturing Annealing/Elongation	10 5 240	96 T_A [a] 60	25
Cooling	∞	4	1

[a] The annealing temperature is specific for each primer and indicated in Table 4

The reactions were further analyzed in an ABI-Prism 3130xl genetic analyzer.

2.2.13 Molecular and phylogenetic analysis

Sequences of influenza A and B viruses, and adenoviruses were first assembled into consensus sequences using the SeqMan Pro software of the Lasergene 10 Coresuite software package (DNAstar, Madsion, WI). Then multiple sequence alignments HA and NA consensus sequences from influenza viruses A(H1N1)pdm09, A(H3N2) and B/Victoria-lineage were

carried out with BioEdit Sequence Alignment Editor 7.2.5. Multiple sequence alignments of the fusion and hexon gene consensus sequences of adenoviruses were also carried out with BioEdit. Multiple sequence alignment of RSV G, HMPV F, and RV VP4/2 gene sequences were compiled using ClustalW in MEGA 5.2, 6.06, and 7.0.14, respectively.

Phylogenetic trees of the influenza virus HA and NA genes were constructed in MEGA 5.2, using the neighbor joining algorithm and the Kimura 2-parameter model. RSV and HMPV phylogenetic tree analyses were performed using the maximum-likelihood algorithm and models Tamura-Nei (TN93+G) for RSV-A, Hasegawa-Kishino-Yano (HKY+G) for RSV-B and Tamura 3-parameter (T92+G) for HMPV. The phylogenetic trees for HAdV and RV were constructed using the neighbor-joining and maximum-composite-likelihood methods. The reliability of the branching order was each estimated by performing 1,000 bootstrap replicates except for RV species A, B, and C; for these 100 bootstrap replicates were performed. The trees were manually edited in Microsoft PowerPoint 2010 and Corel Draw 12 program. Deduced amino acid sequences were translated with the standard genetic code using Bioedit software program.

Pairwise nucleotide and amino acid distances within and between groups were calculated using MEGA and determined distances described in terms of the average mean percentages and range. To estimate the numbers of potentially N-and O-glycosylated residues, the NetNGlyc 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc/) and NetOGlyc 3.1 (http://www.cbs.dtu.dk/services/NetOGlyc-3.1/) servers were respectively used. Putative N-glycosylation site was predicted according to amino acid motif NXT, where X is not proline. Potential O-glycosylation of serine and threonine residues was predicted using a G-score ≥0.5. Synonymous and nonsynonymous mutations were analyzed by the SNAP v2.1.1 program on the human immunodeficiency virus (HIV) sequence database website (http://www.hiv.lanl.gov/content/sequence/SNAP/SNAP.html).

2.2.14 Statistical Analysis

The SPSS program version 20 was used for statistical analysis of data. Association of age and respiratory virus group was analyzed using the chi-squared test and the Fisher's exact test. *P*-values < 0.05 were considered statistically significant. Association of respiratory viruses to clinical presentations was analyzed using odds ratio and 95% confidence interval (CI) [236]. Probit analysis was performed to determine the HPIV assay's limit of detection (95% CI).

3 Results

From February to November 2006 and January 2013 to December 2014, a prospective hospital based study was carried out on children presenting with ALRI at two study hospitals in Accra, Ghana. The hospitals involved were the Korle-Bu Teaching Hospital (KBTH) and the Princess Marie Louise Children's Hospital (PMLCH). The KBTH is a tertiary and national referral hospital which receives patients from all over the country. The PMLCH is a primary health care facility which receives patients mainly from the Greater Accra region and other neighboring regions in the southern part of the country. Patients were enrolled after parents gave a written inform consent. One sample per patient was obtained and averagely 17 samples per month were recorded.

Comprehensive virological screening was performed for 552 respiratory samples for 16 common respiratory viruses including influenza virus type A and B, RSV group A and B, HMPV, HAdV, RV, HPIV type 1 to 4, HCoV type 229E, NL63, OC43 and HKU1, and HBoV, using specific real-time PCR assays. Additionally, phylogenetic analyses were performed to identify circulating virus types, subtypes and genotypes. On these bases, prevalence, seasonal circulation pattern, as well as virus specific associations to age or clinical symptoms have been analyzed.

3.1 Study Location

Ghana is located in West Africa along the coast of the Gulf of Guinea (Fig.9a). The country has 10 administrative regions (Fig.9b); three regions to the north and seven to the southern part of Ghana. The two study hospitals are situated in the nation's capital city Accra, which is within the Greater Accra Region.

Ghana has a primary, secondary and tertiary public health service delivery. In 2002, the Ghana Health Service adopted the integrated disease surveillance and response strategy for Africa [237]. The priority diseases required for reporting include influenza, malaria and HIV/AIDS amongst others. Influenza virus epidemiology in Ghana and Africa at large is not well understood. The National Influenza Center for Ghana, in close collaboration with the Disease Surveillance Department of the Ghana Health Service, operates sentinel surveillance for influenza-like illness (ILI) in 22 sites throughout Ghana, with support from the US

NAMRU3, CDC and WHO [238]. In addition, influenza virus surveillance among SARI patients is ongoing in three selected sentinel sites [239]. Aside from influenza viruses, there are currently no surveillance activities to epidemiologically monitor the circulation and contribution of other common respiratory viruses to the burden of ALRI, particularly in children in Ghana.

(a) (b)

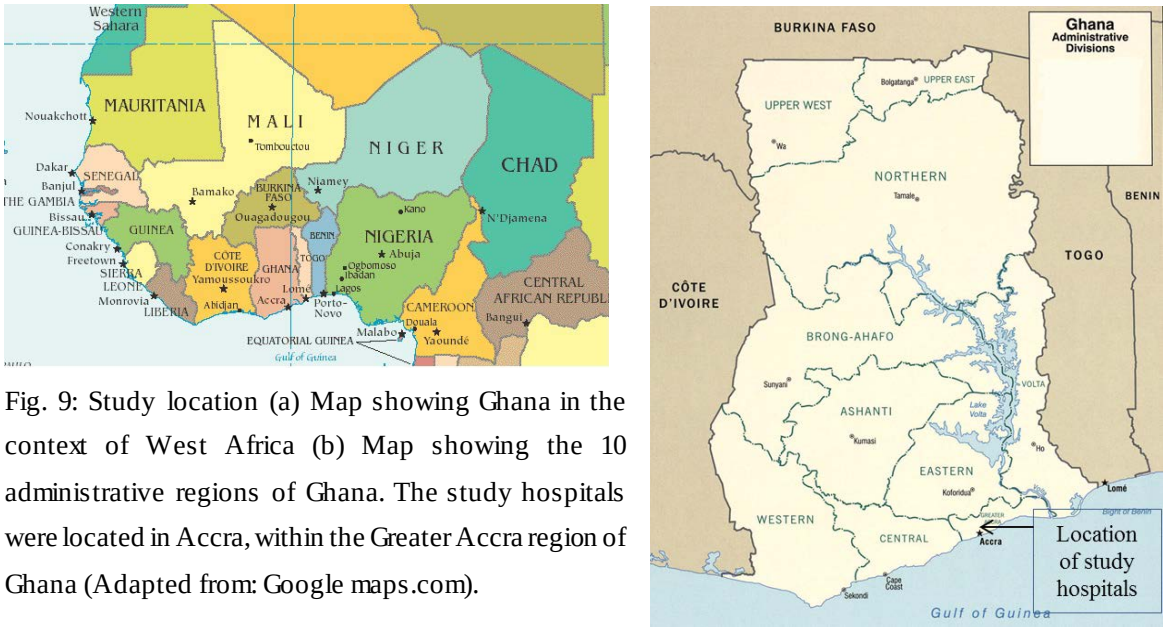


Fig. 9: Study location (a) Map showing Ghana in the context of West Africa (b) Map showing the 10 administrative regions of Ghana. The study hospitals were located in Accra, within the Greater Accra region of Ghana (Adapted from: Google maps.com).

3.2 Demographic characteristics of patients

In this study, a total of 552 children with ALRI were enrolled. Patients were between zero and five years old (Table 8). The majority (53%) of patients were less than one year old. The least (3%) of patients belonged to the age group 4-5 years. The median age was 11 months and 60% were boys. Of the 552 patients, 77% were inpatients. All patients were residents in the southern part of Ghana, with the majority (88%) living in Greater Accra region where the two study sites were located (Fig. 9b).

Table 8: Demography of study participants

Patient's Characteristics (n = 552)	Number (%)
Age in years	
<1	295 (53)
1-2	132 (24)
2-3	76 (14)
3-4	31 (6)
4-5	18 (3)
Median age in month (range)	11(0-59)
Sex	
Girls	222 (40)
Boys	330 (60)
Site of admission in Accra	
Korle-Bu Teaching Hospital	
Inpatients	97 (17)
Princess Marie Louise Children's Hospital	
Inpatients	330 (60)
Out-patients	125 (23)
Geographical Area	
Greater Accra Region	488
Central Region	43
Eastern Region	7
Volta Region	4
Ashanti Region	4
Western Region	3
Brong Ahafo Region	3

3.3 Clinical characteristics of patients

During the patient enrollment process, a questionnaire was administered to parents or guardians to gather basic demographic information and document clinical manifestations of patients. Eight clinical symptoms including fast breathing, cough, nasal discharge, fever, difficulty in breathing, difficulty in feeding, vomiting, diarrhea and abdominal pain were recorded. Additionally patient diagnoses were classified into five main categories including bronchopneumonia, pneumonia, bronchiolitis, respiratory distress and respiratory tract infection (RTI). RTI referred to patients who had both an upper respiratory tract infection and an unclassified lower respiratory tract infection. Further, other diseases such as bronchitis, tonsillitis and otitis media were diagnosed. The clinical data showed that patients presented with more than one symptom, and at least one clinical diagnosis was ascertained for every patient. Frequently reported symptoms were cough (n = 492; 89%), nasal discharge (n = 398;

72%), fever (n = 292; 53%) and difficulty-in-breathing (n = 240; 43%) amongst others (Fig. 11a). The major clinical diagnosis was bronchopneumonia (n = 304; 55%), followed by RTI (n = 135; 24%) and pneumonia (n = 88; 16%) (Fig. 10b). In addition to ALRI, 53 (10%) comorbid conditions were observed among the patients. Malaria (38%) and skin sepsis (34%) contributed to more than two-thirds of comorbidities (Fig. 10c).

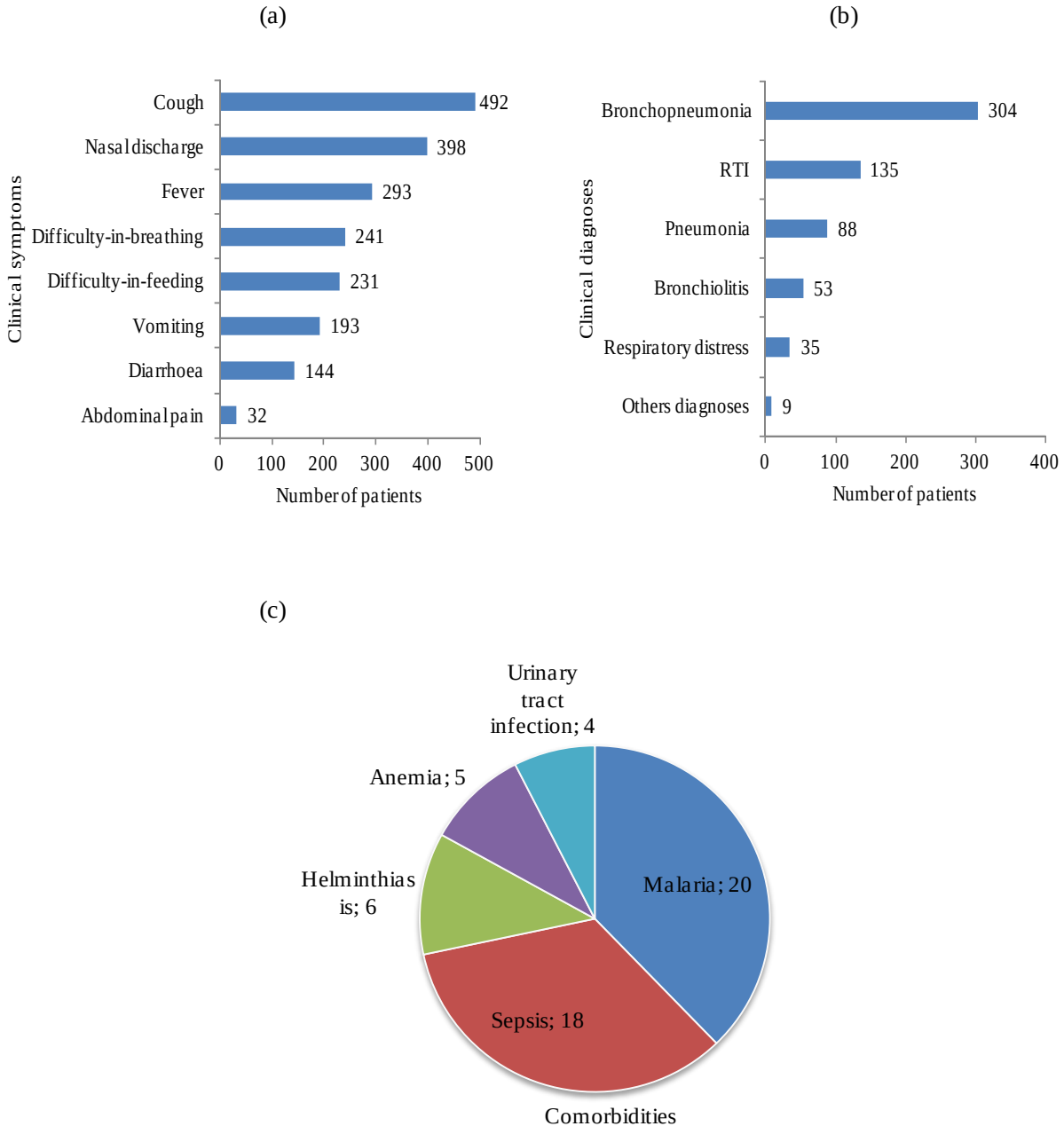


Fig. 10: Clinical manifestations of patients (a) clinical symptoms, (b) diagnoses and (c) comorbidities

3.4 Prevalence and coinfections of respiratory viruses

A total of 552 nasopharyngeal aspirates and nasal swabs were investigated for a panel of 16 respiratory viruses including influenza A and B viruses, RSV group A and B, HMPV, HAdV, RV, HPIV1-4, HCoV-229E, -NL63, -OC43, -HKU1, and HBoV using specific real-time PCR assays. The distribution of respiratory specimens in the three study years were as follows: 47 samples in 2006, 365 in 2013, and 140 in 2014. Overall, 404 (73%) samples were positive for at least one virus (Table 9). RSV (23%) was the most frequent respiratory virus detected, followed by RV (19%), HBoV (14%), HPIV (12%) and HAdV (12%). Influenza virus and HCoV were each identified in 6% of the samples. The least detected pathogen was HMPV (3%). The prevalence for each pathogen varied annually, with RSV being the most prevalent every year. Except for influenza virus, all respiratory viruses were detected in each year (Table 9).

Table 9: Detection of respiratory viruses in patients with ALRI

Year	Number of samples	Number (% of positive samples)	Number (% viral pathogens detected)							
			RSV	RV	HBoV	HPIV	HAdV	IV	HCoV	HMPV
2006	47	29 (62)	13 (28)	13 (28)	2 (4)	1 (2)	3 (6)	0 (0)	2 (4)	1 (2)
2013	365	278 (76)	84 (23)	74 (20)	49 (13)	45 (12)	49 (13)	29 (8)	20 (6)	9 (3)
2014	140	97 (69)	30 (21)	15 (11)	28 (20)	19 (14)	12 (9)	6 (4)	7 (5)	7 (5)
Total	**552**	**404 (73)**	**127 (23)**	**103 (19)**	**79 (14)**	**65 (12)**	**64 (12)**	**35 (6)**	**25 (6)**	**17 (3)**

Single and multiple virus infections were evaluated (Table 9). Of the 404 positive samples, 306 (76%) single viral infections and 98 (24%) multiple viral infections were identified. The multiple infections included 85 (21%) double infections and 13 (3%) triple infections. RSV, RV, HPIV and influenza virus were more commonly identified as single pathogen. Coinfections were observed for the majority of respiratory viruses, with RV and HBoV been detected in most of the coinfections. However no coinfection was observed between HPIV and influenza virus, influenza virus and HMPV, and HMPV and HCoV (Table 10). The combination of viruses involved in triple infection is shown in Fig. 11.

Table 10: Determination single and multiple virus detections

Virus	N (%)	RSV	RV	HBoV	HPIV	HAdV	IV	HCoV	HMPV
RSV		**96**	12	5	2	6	1	7	3
RV		-	**56**	19	4	14	2	1	4
HBoV		-	-	**33**	7	15	6	2	1
HPIV		-	-	-	**49**	3	0	2	1
HAdV		-	-	-	-	**28**	3	2	1
IV		-	-	-	-	-	**24**	1	0
HCoV		-	-	-	-	-	-	**12**	0
HMPV		-	-	-	-	-	-	-	**8**
Infection									
Single	**306 (76)**	**96**	**56**	**33**	**49**	**28**	**24**	**12**	**8**
Double	**85 (21)**	26	38	37	13	28	9	10	8
Triple	**13 (3)**	5	9	9	3	8	1	3	1
Total	**404 (100)**	127	103	79	65	64	35	25	17

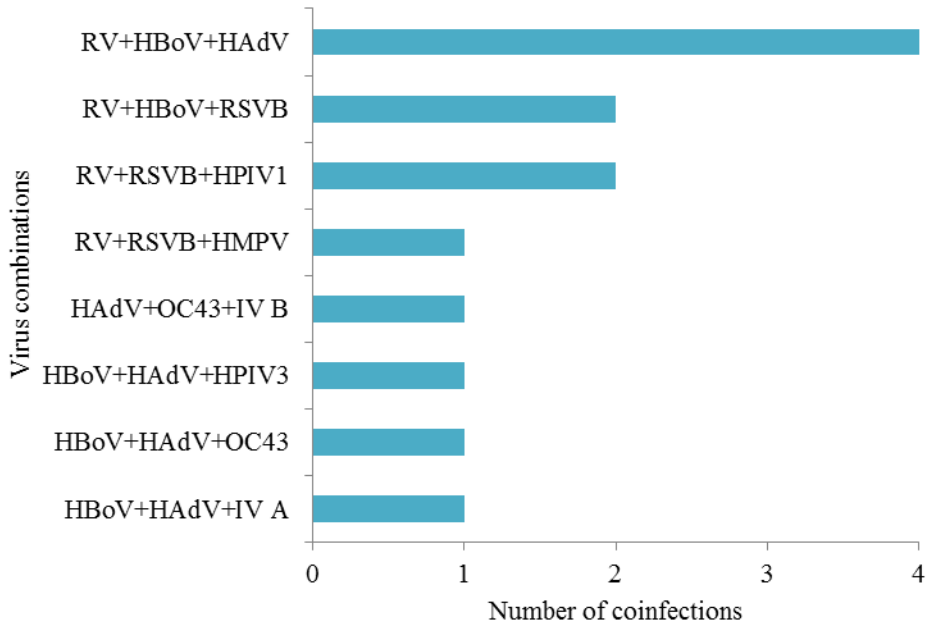


Fig. 11: Virus combinations of triple infection

3.5 Association of respiratory viruses to age groups

The frequency of respiratory virus infection was analyzed with relation to the age of children (Table 11). Three age groups were studied. The infant group represented children less than one year old; the toddler group children between 1-3 years old, and the pre-school group

children between 3-5 years old. The association between the viral pathogens and different age groups was statistically analyzed using the chi-squared test and Fisher`s exact t-test. RSV (33%; $p \leq 0.0001$) was significantly associated with the infant group, HBoV (21%, $p = 0.007$) with the toddler group, and influenza virus (22%; $p \leq 0.0001$) with the pre-school group (Table 11). Moreover RV was most prevalent among toddlers (25%) and was only second (17%) to RSV infection among infants. HMPV was only observed in the between 0-2 years old patients.

Table 11: Detection of respiratory viruses by age group

	Number (% of virus-positive samples)					
	Infants	**Toddlers**		**Pre-school**		
	< 1yr	**1-2yrs**	**2-3yrs**	**3-4yrs**	**4-5yrs**	
Virus	**n = 295**	**n = 132**	**n = 76**	**n = 31**	**n = 18**	***p*-value**
RSV	**97 (33)**	16 (12)	13 (17)	0	1 (6)	**≤ 0.0001**[a]
RV	50 (17)	28 (21)	19 (25)	4 (13)	2 (11)	0.377
HBoV	27 (9)	**28 (21)**	**16 (21)**	5 (16)	3 (17)	**0.007**[a]
HPIV	37 (13)	17 (13)	7 (9)	3 (10)	1 (6)	0.835
HAdV	30 (10)	21 (16)	7 (9)	4 (13)	2 (11)	0.481
IV	8 (3)	14 (11)	5 (7)	4 (13)	**4 (22)**	**≤ 0.0001**[b]
HCoV	16 (5)	5 (4)	1 (1)	3 (10)	0	0.283
HMPV	12 (4)	5 (4)	0	0	0	0.380

[a] *p*-value was calculated using the Chi-squared test

[b] *p*-value was calculated using the Fisher`s exact t-test

3.6 Correlation of respiratory pathogens with clinical presentations

To correlate the clinical symptoms and diagnoses with the identified viruses, an unadjusted odds ratio (OR) analyses with a 95% confidence interval (CI) was used [236]. RSV infection was associated with cough (OR 2.9; 95% CI = 41.23 to 6.99), nasal discharge (OR 1.67; 95% CI = 1.04 to 2.70) and difficulty in breathing (OR 1.90; CI = 1.27 to 2.84) (Table 12). Patients infected with RSV further had diagnosis of bronchopneumonia (OR 4.25; 95% CI = 2.65 to 6.81) and bronchiolitis (OR 2.02; 95% CI = 1.11 to 3.69). RV infection was associated with

cough (OR 2.79; 95% CI =1.09 to 7.16) and pneumonia (OR 1.70; 95% CI = 1.00 to 2.89). HBoV infection correlated well with nasal discharge (OR 2.68; 95% CI = 1.38 to 5.22). HPIV infection was associated with fever (OR 2.19; 95% CI = 1.25 to 3.81). HAdV infection was associated with diarrhea (OR 1.84; 95% CI = 1.07 to 3.18) and RTI (OR 1.76; 95% CI = 1.01 to 3.07). Influenza virus infection correlated with cough (OR 14.96; 95% CI = 2.03 to 110.3), nasal discharge (OR 4.40; 95% CI = 1.33 to 14.59) and RTI (OR 4.11; 95% CI = 2.05 to 8.24). Surprisingly, fever was not associated with influenza virus infection (OR 1.97; 95% CI = 0.94 to 4.10). HMPV was associated with diarrhea (OR 4.28; 95% CI = 1.60 to 11.45).

3.7 Circulation of respiratory viruses

Ghana has a tropical climate with two main seasons: Wet or rainy season and dry season [240]. The wet season is generally cool with temperatures between 21^{o}C and 28^{o}C. The rains last from April to October. The rest of the year from November to March is generally dry and hot weather with temperatures up to 38^{o}C.

RSV had a strong seasonal activity during the wet season that started around June/July and gradually peaked in October of each season (Fig. 12). HMPV showed higher activity in June/July. However the peak activity of both pathogens does not necessarily coincide with an increase amount of rainfall. Influenza virus, HCoV and HAdV circulated sporadically year-round without a marked seasonality. HPIV, RV and HBoV circulated year-round but predominantly during the dry season with the highest activity in February and March of each year, excluding 2006. Co-circulation of viruses was common during the dry seasons as well as in the wet seasons.

Table 12: Analysis of respiratory viruses according to clinical manifestations

Clinical manifestation	OR (95% CI)[a]							
	RSV	**RV**	**HBoV**	**HPIV**	**HAdV**	**IV**	**HCoV**	**HMPV**
Symptom	**2.94**	**2.79**	1.30	2.74	1.20	**14.96**	0.04	0.91
Cough	**(1.23-6.99)**	**(1.09-7.16)**	(0.57-2.97)	(0.83-9.02)	(0.50-2.92)	**(2.03-110.3)**	(0.01-0.10)	(0.20-4.09)
	1.67	0.88	**2.68**	1.21	1.61	**4.40**	0.98	1.27
Nasal discharge	**(1.04-2.70)**	(0.55-1.40)	**(1.38-5.22)**	(0.67-2.20)	(0.85-3.05)	**(1.33-14.59)**	(0.40-2.39)	(0.41-3.95)
	0.84	0.98	1.37	**2.19**	0.94	1.97	0.39	0.60
Fever	(0.57-1.25)	(0.64-1.50)	(0.84-2.22)	**(1.25-3.81)**	(0.56-1.59)	(0.94-4.10)	(0.17-0.93)	(0.22-1.59)
	1.90	1.11	0.97	0.38	1.33	0.57	1.01	0.53
Difficulty- breathing	**(1.27-2.84)**	(0.72-1.71)	(0.60-1.57)	(0.21-0.69)	(0.79-2.25)	(0.27-1.19)	(0.45-2.28)	(0.18-1.52)
	1.10	1.21	1.11	0.83	**1.84**	0.69	0.37	**4.28**
Diarrhea	(0.71-1.72)	(0.75-1.94)	(0.65-2.68)	(0.45-1.53)	**(1.07-3.18)**	(0.30-1.62)	(0.11-1.27)	**(1.60-11.45)**
Diagnosis	**4.25**	0.83	0.64	0.77	0.60	0.40	0.88	2.73
Bronchopneumonia	**(2.65-6.81)**	(0.54-1.27)	(0.40-1.03)	(0.46-1.29)	(0.36-1.02)	(0.20-0.83)	(0.39-1.96)	(0.88-8.47)
	0.46	**1.70**	1.16	1.22	0.51	0.67	0.71	1.65
Pneumonia	(0.24-0.88)	**(1.00-2.89)**	(0.62-2.18)	(0.62-2.40)	(0.21-1.23)	(0.23-1.93)	(0.21-2.42)	(0.53-5.19)
	2.02	1.84	0.60	0.59	1.18	0	1.86	1.27
Bronchiolitis	**(1.11-3.69)**	(0.97-3.60)	(0.23-1.55)	(0.20-.166)	(0.51-2.74)	(0)	(0.61-5.63)	(0.28-5.69)
	0.43	0.55	1.64	1.21	**1.76**	**4.11**	1.48	0.40
Respiratory tract infection	(0.25-0.75)	(0.32-0.97)	(0.98-2.74)	(0.68-2.17)	**(1.01-3.07)**	**(2.05-8.24)**	(0.62-3.52)	(0.09-1.79)

[a] OR: Odds ratio; CI: Confidence interval

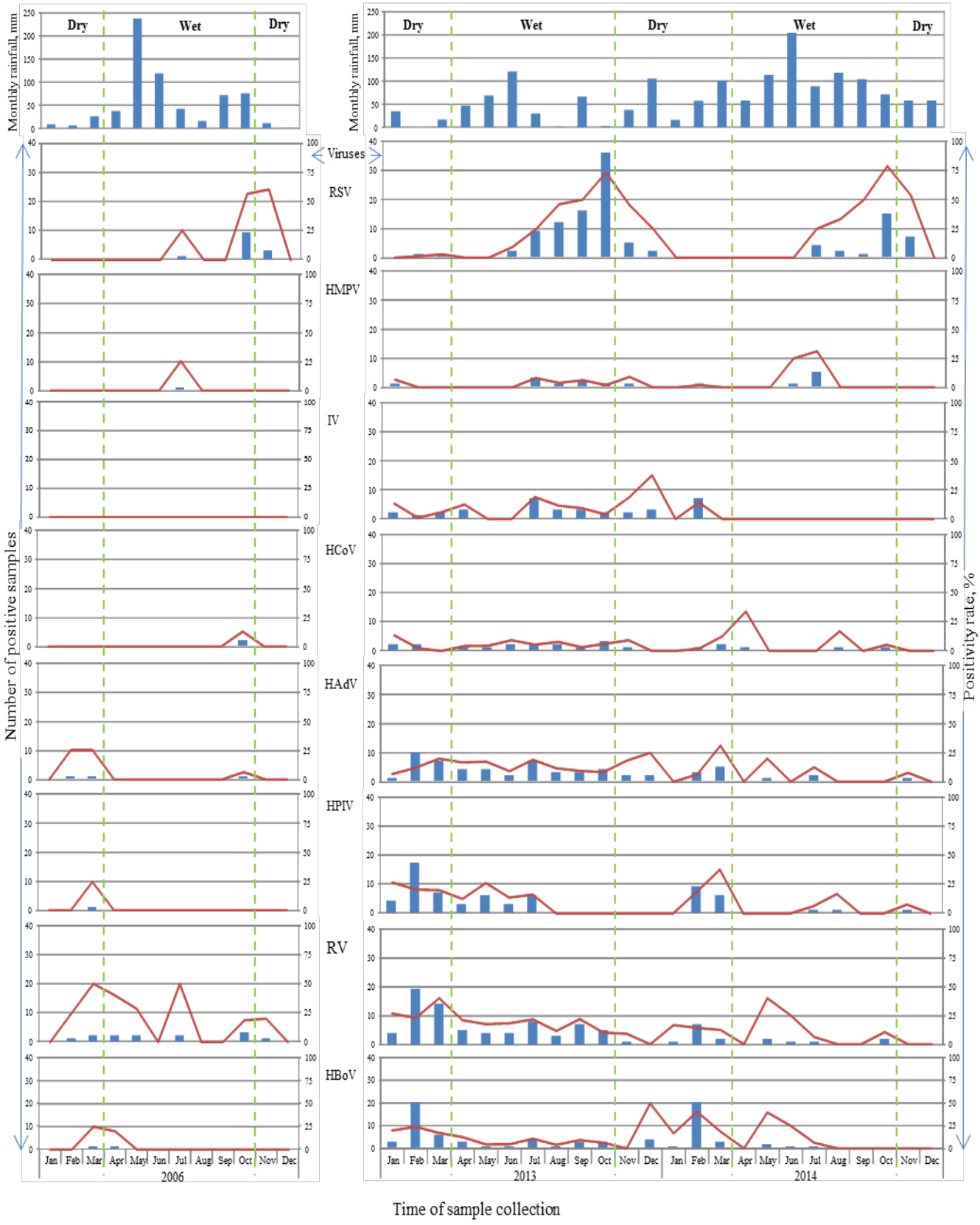


Fig. 12: Seasonal circulation patterns of respiratory viruses. Monthly rainfall for Greater Accra Region included illustrating the dry and wet seasons (source of the rainfall data: Ghana Meteorological Agency).

Abbreviations: IV = influenza virus; RSV = Respiratory syncytial virus; HMPV = Human metapneumovirus; RV = Human rhinovirus; HAdV = Human adenovirus; HBoV = Human Bocavirus; HPIV = Human parainfluenza virus; HCoV = Human coronavirus.

3.8 Differentiation of respiratory viruses into types, subtypes, lineages or groups

3.8.1 Differentiation of influenza A and B viruses

Of 552 samples investigated, 35 samples tested positive for influenza viruses by real time PCR. The influenza viruses were detected in samples from 2013 and 2014 (Table 13). Differentiation of the influenza viruses by specific real-time PCR revealed that influenza virus activity was primarily related to influenza A(H3N2) virus (63%), and B/Victoria-lineage viruses (31%), whereas influenza A(H1N1)pdm09 virus accounted for only 6% of circulating influenza viruses. Influenza viruses were detected throughout the year 2013 with higher activities in April, July and December (Fig. 13). The peak activity in July was the only time when the two influenza A virus subtypes and influenza B viruses were simultaneously identified. The missing detections in May and June reflect the low level of influenza virus activity during these months. Influenza A(H3N2) virus was the dominant subtype in 2013. In 2014 influenza viruses were detected in February and coincided with the simultaneous circulation of the two influenza A virus subtypes and B/Victoria- lineage predominating.

Table 13: Distribution of influenza virus types and subtypes

			Number (% of influenza virus types/subtypes		
Year	**Number of sampes**	**Number of IV-positives**	**A(H3N2)**	**A(H1N1)pdm09**	**B/Victoria-lineage**
2006	47	0	0	0	0
2013	365	28	20 (71)	1 (4)	7 (25)
2014	140	7	2 (29)	1 (14)	4 (57)
Total	**552**	**35**	**22 (63)**	**2 (6)**	**11 (31)**

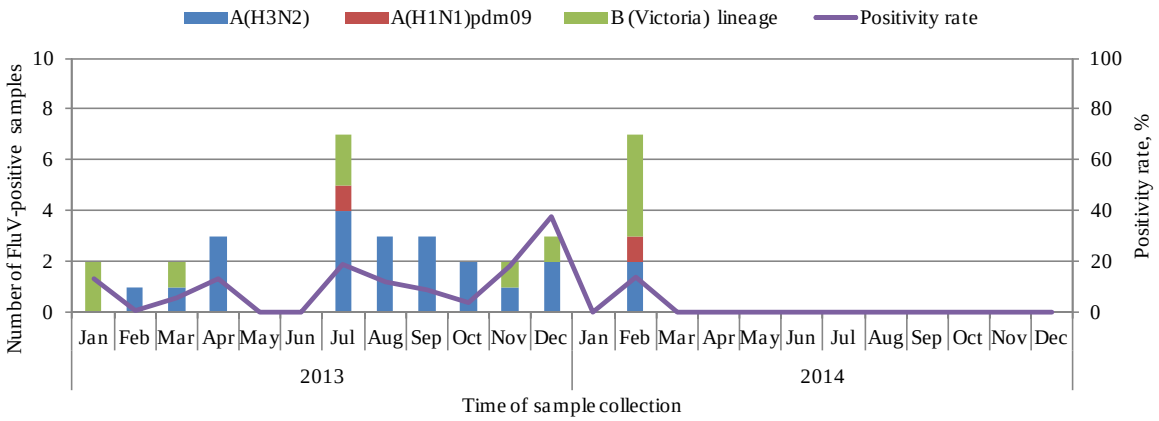


Fig. 13: Circulation patterns of influenza A and B viruses

3.8.1.1 Antigenic analyses of influenza A and B viruses

MDCK-SIAT cells were infected with a total of 35 respiratory samples previously identified to be influenza virus-positive by real time PCR. Of the 35 PCR-positive influenza virus samples, 18 could be identified in cell culture as influenza virus isolates by the HA test (Table 14). The influenza virus-positive cultures were adjusted to a titer of 4HA units/50µl and further analyzed by the hemagglutination inhibition (HI) test to determine their antigenic characteristics (see 2.2.3 and 2.2.4). Four isolates had low titers of 2HA units/50µl and therefore could not be antigenically characterized. The HI test was performed using panels of specific post-infection ferret antisera raised against vaccine strains recommended by WHO for seasons 2012-2013 and 2013-2014. These included the antiserum against the vaccine strain A/California/7/09 which was used for analysis of A(H1N1)pdm09 isolates, and the antiserum against the vaccine strain A/Texas/50/2012 which was used for the analysis of A(H3N2) isolates. Moreover, since A/Switzerland/9715293/13 emerged as a new A(H3N2) drift variant, the antiserum against this strain was also included. Influenza B virus isolates were analyzed using antisera raised against B/Brisbane/60/2008 and B/Massachusetts/2/2012 vaccine strains and further with antiserum raised against the new reference strain B/Phuket/3073/13 (Table 14). From the 14 isolates antigenically characterized the virus types identified were as follows: eight influenza A(H3N2), two A(H1N1)pdm09, and four B/Victoria-lineage viruses. Compared with the titer obtained with the antisera to the homologous virus, about 50% of virus isolates were recognized by their corresponding antisera within 4-fold of the titer with the homologous virus. The influenza A(H1N1)pdm09 viruses remained antigenically homogeneous to the vaccine virus strain A/California/7/09. The majority of influenza A(H3N2) isolates showed an 8- or 16- fold reduction compared to the vaccine strain A/Texas/50/2012, indicating a profile antigenically different from the vaccine strain. None of the influenza A(H3N2) isolates reacted with the antiserum raised against the new reference strain A/Switzerland/9715293/13. Three of the four influenza B isolates reacted well with antisera to the vaccine strain B/Brisbane/60/2008 of the Victoria-lineage. Nonetheless, no reactivity of the isolated influenza B viruses was seen with antisera raised against the B/Massachusetts/2/2012 vaccine strain, or the new reference strain B/Phuket/3073/13 representing the B/Yamagata-lineage (Table 14).

Table 14: Antigenic analysis and HI titer of influenza and B viruses

		Post-infection ferret antisera					
		H3		H1	B		
Virus isolates	HA titer	Tex12	Swit13	Cal09	Bris08	Mass12	Phuk13
Vaccine strain							
A/Texas/50/2012		1280	320	<20			
A/Switzerland/9715293/13		160	1280	<20			
A/California/7/09		<20	<20	640			
B/Brisbane/60/2008					640	<20	<20
B/Massachusetts/2/2012					<20	320	320
B/Phuket/3073/13					<20	160	320
A(H3N2)							
GHA/RV084/2013	2	ND	ND	ND			
GHA/RV121/2013	2	ND	ND	ND			
GHA/RV133/2013	2	ND	ND	ND			
GHA/RV147/2013	8	80	<20	<20			
GHA/RV208/2013	8	80	<20	<20			
GHA/RV215/2013	32	80	<20	<20			
GHA/RV236/2013	32	160	<20	<20			
GHA/RV262/2013	64	320	<20	<20			
GHA/RV263/2013	64	320	<20	<20			
GHA/RV285/2013	64	160	<20	<20			
GHA/RV307/2013	2	ND	ND	ND			
GHA/RV359/2013	32	80	<20	<20			
A(H1N1)pdm09							
GHA/RV219/2013	64	<20	<20	160			
GHA/RV004/2014	32	<20	<20	160			
B/Victoria-lineage							
GHA/RV348/2013	32				80	<20	<20
GHA/RV023/2014	32				160	<20	<20
GHA/RVk016/2014	128				160	<20	<20
GHA/RVk020/2014	128				160	<20	<20

Cal09 (A/California/7/09), Tex12 (A/Texas/50/2012), Swit13 (A/Switzerland/9715293/13), Bris08 (B/Brisbane/60/2008), Mass12 (B/Massachusetts/2/2012), Phuk13 (B/Phuket/3073/13), ND – not determined

3.8.2 Differentiation of RSV group A and B viruses

There were 127 samples positive for RSV. Differentiation by specific real-time PCR showed that 49 (39%) were RSV group A and 78 (61%) were RSV group B (Table 15). RSV group B dominated in 2006 (85%), and in 2013 (80%). In 2014 RSV group A predominated and was the sole RSV group circulating. The samples from 2006 were previously investigated for RSV using a traditional conventional RT-PCR method [23]. The results were confirmed with the sensitive and specific real-time PCR assay.

Table 15: Distribution of RSV group A and B viruses

Year	Number of specimens	Number (% of RSV-positive specimens)	Number (% RSV A)	Number (% RSV B)
2006	47	13 (28)	2 (15)	11 (85)
2013	365	84 (23)	17 (20)	67 (80)
2014	140	30 (21)	30 (100)	0
Total	**552**	**127 (23)**	**49 (39)**	**78 (61)**

Even though yearly RSV circulation in the wet season was commonly from June/July to November/December, co-circulation of group A and B with peak activity in October 2006 and September-November 2013 was observed. No co-circulation was observed in 2014 (Fig. 14).

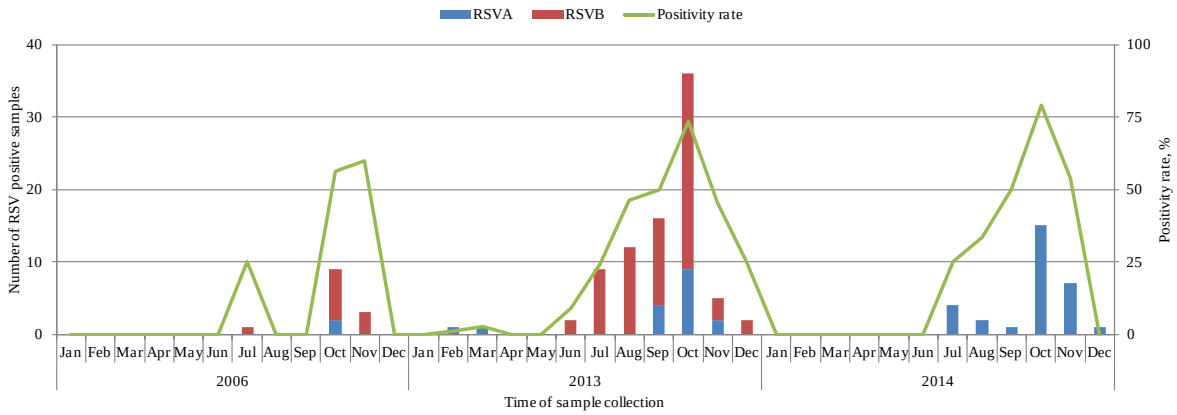


Fig. 14: Seasonal and monthly distribution of RSV group A and B viruses

3.8.3 Genotyping of HAdV

During the three-year study period from 2006, 2013 and 2014, sixty-four children were identified with HAdV respiratory infection. The samples obtained from these infections were further characterized according to the causative HAdV species using the fluorescence melting curve analysis. The analysis shows the circulation of HAdV species A to F (Table 16). Of these, the most prevalent species were HAdV C (n= 28, 43.8%) and HAdV B (n = 21, 32.8%). The detection rates for both species were highest in children less than 2 years (data not shown). HAdV species D and F were each detected in 4 (6.3%) HAdV-positive samples, while HAdV species E and F were each detected in 3 (4.7%) of HAdV-positive samples. The number of

occurring HAdV species varied with the number of HAdV positives per year. In 2006 the three HAdV positives identified belonged to species C. In 2013 all HAdV species were identified amongst a total of 49 samples and in 2014 species B, C, D and F were detected amongst 11 samples.

Table 16: Distribution of HAdV species

HAdV species	Number (% of HAdV positives)	Number of HAdV species		
		2006	2013	2014
A	3 (4.7)	0	3	0
B	21 (32.8)	0	16	5
C	28 (43.7)	3	21	4
D	4 (6.3)	0	3	1
E	3 (4.7)	0	3	0
F	4 (6.3)	0	3	1

3.8.4 Genotyping of HPIV

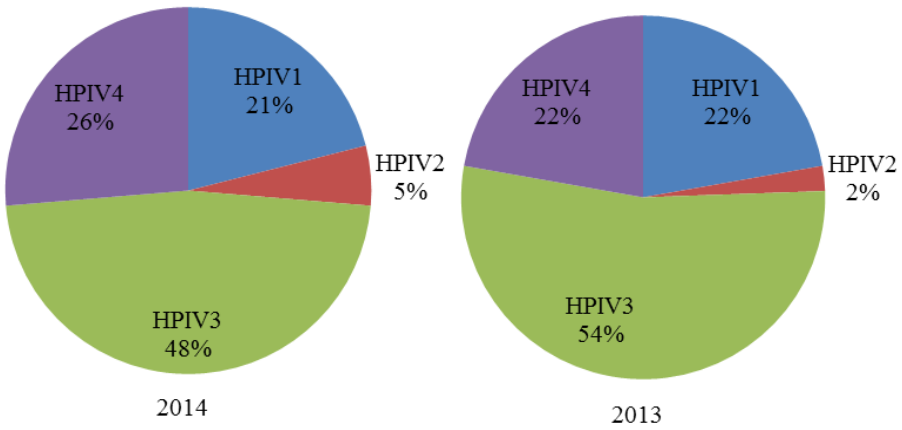
A total of 65 HPIV-positive samples were differentiated into HPIV types by the real time quadruplex PCR assay. Among these, the majority were HPIV3 (n = 33, 51%) followed by HPIV4 (n = 15, 23%), HPIV1 (n = 14, 22%), and HPIV2 (n = 3, 5%). Patients less than two years old were mostly affected by all four HPIV types and primarily by HPIV3 (Table 17). The detection rate of HPIV in children between 2-5 years old was low and therefore an association to certain HPIV type was not possible.

Similar circulation pattern and co-circulation of all HPIV types in the seasons 2013 and 2014 was observed (Fig. 15a). HPIV3 and HPIV4, the two most frequently detected types predominantly drove these seasonal trends. There was only one HPIV-positive sample (HPIV2) detected in season 2006 (data not shown). Whereas the proportion of HPIV types is equally distributed in 2013 and 2014, the timely circulation of these types is slightly different (Fig. 15b).

Table 17: Distribution of HPIV types across age group

Age group in years	Number of HPIV patients	Number (% of HPIV types)			
		HPIV1	HPIV2	HPIV3	HPIV4
<1	37	6 (16)	1 (3)	19 (51)	11 (30)
1-2	17	5 (29)	1 (6)	9 (53)	2 (12)
2-3	7	3 (43)	1 (14)	3 (43)	0
3-4	3	0	0	1 (33)	2 (67)
4-5	1	0	0	1 (100)	0
Total	**65**	**14 (22)**	**3 (5)**	**33 (51)**	**15 (23)**

(a)



(b)

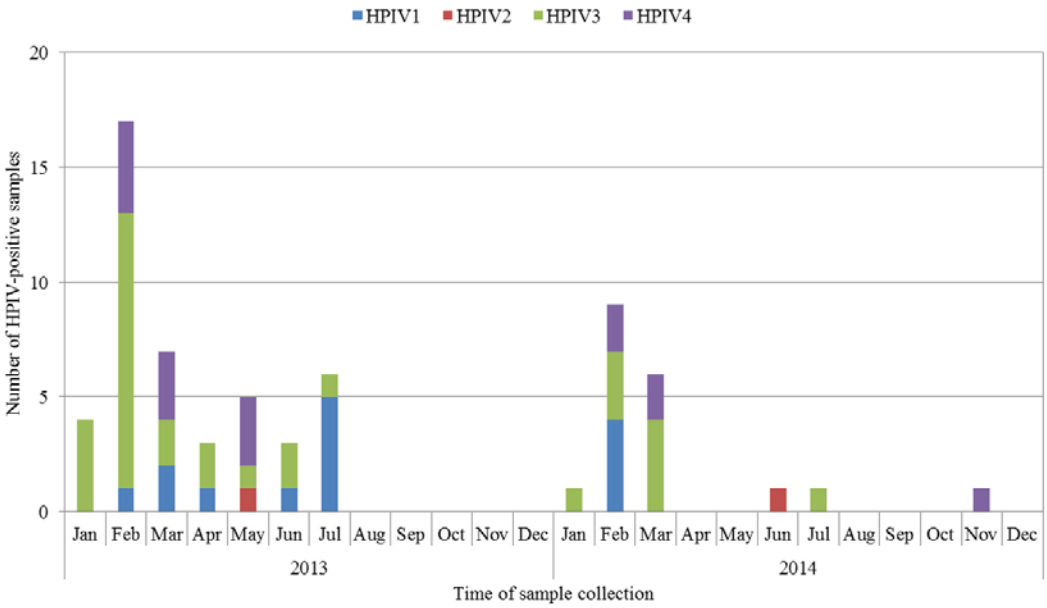


Fig. 15: Distribution of the HPIV types (a) by year and (b) by month

3.8.5 Genotyping of HCoV

A multiplex real-time PCR was performed for the detection of the four HCoV species; HCoV-229E, -OC43, -NL63 and -HKU1. Of the 25 HCoV-positive samples identified, OC43 was the most prevalent (9, 36%) followed by 229E (8, 32%), HKU1 (6, 24%) and NL63 (3, 12%) (Table 18). One sample was infected with both 229E and HKU1. The yearly distribution of the HCoV varied according to each species. HKU1 was detected in all three study years, while NL63 was detected only in 2013. 229E and OC43 were both detected in 2013 and 2014.

Table 18: Distribution of HCoV species

Year	Number of samples tested	Number of positive samples	Number (% HCoV species)			
			OC43	229E	HKU1	NL63
2006	47	2	0	0	2 (100)	0
2013	365	17	6 (35)	6 (35)	2 (12)	3 (18)
2014	140	6	3 (50)	2 (33)	2 (33)	0
Total	**552**	**25**	**9 (36)**	**8 (32)**	**6 (24)**	**3 (12)**

3.9 Molecular characterization of circulating respiratory viruses in ALRI

3.9.1 Influenza viruses

In order to study the evolution and epidemiology of influenza viruses circulating recently, the hemagglutinin (HA) and neuraminidase (NA) genes of all the 35 influenza virus-positive samples from this study were sequenced and genetically analyzed. Influenza viruses which generated poor sequences probably due to low virus copy numbers in sample were excluded from the phylogenetic analyses. A total of 18 influenza virus-positive samples were aligned and compared with other representative viruses identified in Ghana since 2009, as well as WHO reference vaccine strains and viruses circulating in other regions of the world. Apart from sequences from this study, the other reference sequences used in the analyses were available in GISAID (the Global Initiative on Sharing All Influenza Data) database.

3.9.1.1 Phylogenetic analysis of influenza A(H3N2) viruses

The phylogenetic analysis of the HA genes of A(H3N2) viruses revealed seven main genetic groups that evolved between 2009 and 2015 and were characterized by clade specific amino acid substitutions (Fig. 16a). In Ghana, viruses circulating since 2009 fell into different genetic groups and subgroups according to the years or seasons. Viruses which circulated between the seasons 2009 and 2010 acquired the amino acid substitution N144K and belonged to genetic group 1 with the A/Perth/16/2009 reference strain. Viruses circulating between the seasons 2011 and 2012 acquired the amino acid substitution N144K and belonged to genetic group 3A with the A/Stockholm/18/2011 reference strain.

The viruses circulating in the season 2013 including viruses from this study acquired the amino acid substitutions T128A and R142G and fell into the genetic group 3C subgroup 3C.3. This group is represented by the A/Samara/73/2013 and A/South Africa/4655/2013 reference strains. Other viruses from Ghana, Egypt, and for example the Netherlands belong to this group and were closely related to the study viruses. Recent viruses circulating between the seasons 2014 and 2015 including viruses from Ghana fell into the genetic subgroups 3C.3a and 3C.2a respectively. The 3C.3a group is represented by the A/Switzerland/9715293/2013 vaccine strain with the amino acid substitutions A138S, F159S and N225D, while the 3C.2a subgroup is represented by the A/Hong Kong/5738/2014 reference strain (Fig. 16a).
The genetic group 3 is subdivided into groups 3A, 3B and 3C. The genetic group 3C further has three divisions: 3C.1, 3C.2 and 3C.3, and three additional genetic subgroups: 3C.2a (from 3C.2), and 3C.3a and 3C.3b (from 3C.3) which emerged recently in 2014 as a result of antigenic drift.

Three viruses from this study belonging to the genetic group 3C fell into a separate subcluster and carried exclusively the amino acid substitution S124N. However the subcluster is unique and does not belong to the genetic subgroup 3C.3 because they lacked the requisite amino acid substitutions T128A and R142G. Neither does this subcluster belong to the genetic subgroup 3C.2a because they lacked the specific amino acid substitutions L3I, N144S, F159Y, K160T, N225D or Q311H. Such a group of viruses may possibly be considered as intermediates or ancestors of 3C.2a and 3C.3 genetic groups. Besides, an earlier circulation of these intermediate viruses may be suggested. Another virus from Ghana (A/Ghana/DARI-0098/2013) which circulated during 2013 clustered with the intermediate viruses from this study (Fig 16a).

The phylogenetic analysis of the NA genes of A(H3N2) viruses revealed that some viruses from this study showed heterogeneous grouping and do not cluster in the same manner as their corresponding HA genes (Fig. 16b). A group of four viruses (GHA/RV/236/2013, GHA/RV/262/2013, GHA/RV/307/2013, and GHA/RV/359/2013) with HA genes in the genetic group 3C.3 clustered in genetic group 3A of the NA phylogeny indicating an inter-clade reassortment. Similarly the 'ancestor' subcluster of viruses with HA gene in genetic group 3C clustered with genetic subgroup 3C.3 viruses in the NA phylogeny indicating an intra-clade reassortment. It is noteworthy that the "HA 3C.3/NA 3A" inter-clade reassortant viruses carried dual substitutions K220N and K308Q in their NA genes, whilst the "HA 3C/NA 3C.3" intra-clade reassortant viruses carried a unique amino acid substitution D197E in their NA genes, (Fig. 16b).

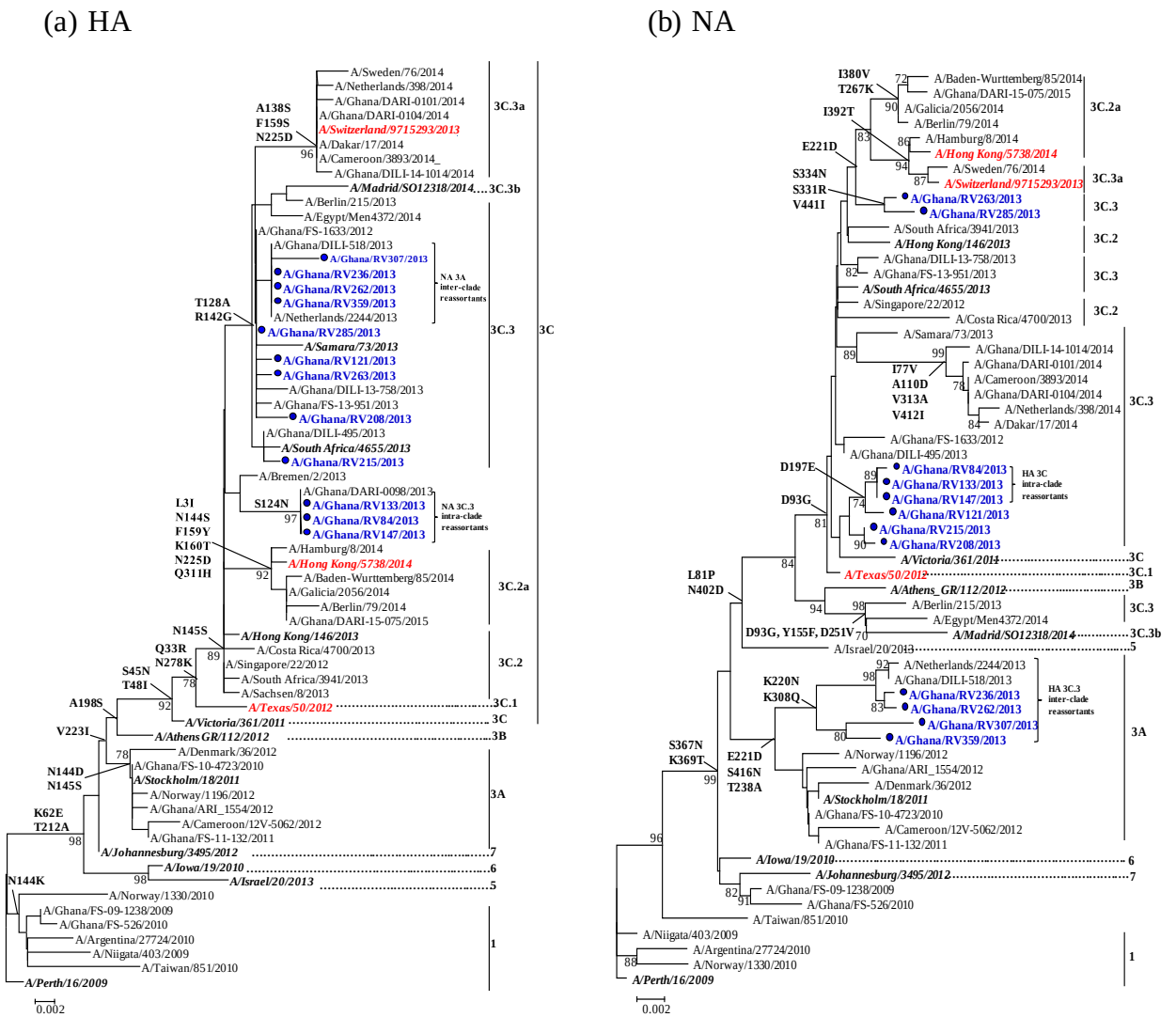


Fig. 16: Phylogenetic analysis of HA and NA genes of influenza A(H3N2) virus (a) HA gene (nt21-nt1140) and (b) NA gene (nt38-nt1430). The neighbor-joining tree was constructed with MEGA version 5.2 using the Kimura-2-parameter model with 1000 bootstrap replicates. Bootstrap values greater than 70% are displayed on branch nodes. Reference sequences of different genetic groups available in GISAID database are indicated by their accession numbers. WHO reference vaccine strains recommended are highlighted in red: A/Texas/50/2012 for the season

2014/15; A/Switzerland/9715293/2013 for the season 2015/16 and A/Hong Kong/5738/2014 for the season 2016/17. Sequences from this study are highlighted in blue and designated by the geographic location (Ghana), patient number and year of collection. Deduced amino acid substitutions are related to the A/Perth/16/2009 reference strain. Clade-specific substitutions and fixed mutations are specified in bold on the left side of node. Clades (1, 3A, 3B, 3C.1, 3C.2, 3C.2a, 3C.3, 3C.3a, 3C.3b, 4, 5, 6, and 7) are designated for both the HA and NA phylogenetic trees.

3.9.1.2 Phylogenetic analysis of influenza A(H1N1)pdm09 viruses

The phylogenetic analysis of the HA genes of A(H1N1)pdm09 viruses revealed a significant genetic drift of these viruses since their emergence in 2009 (Fig. 17a). Until now, eight genetic groups evolved with A/California/7/2009 representing group 1. Influenza A(H1N1)pdm09 viruses which circulated in Ghana over the past few years belonged to different genetic groups. Viruses collected during 2009 and 2010 (pandemic period) do not belong to any designated genetic group and are closely related to the vaccine viral strain A/California/7/2009. A group of viruses from Ghana identified in 2012-2013 segregated in a different subcluster among the 2009-2010 pandemic strains. Although these viruses were still closely related to A/California/7/2009, but the 100% bootstrap value with the unique amino acid changes L32I, D86E, S128T and R259K of the subcluster give an indication of persistence or independent lineage evolution. Viruses from 2011 cluster in the genetic group 8 along with other West Africa strains possessing two group-specific amino acid substitutions, A186T and V272A. A(H1N1)pdm09 viruses from 2012 fell into genetic group 7 denoted by the A/St. Petersburg/100/2011 reference strain, and characterized by S143G and A197T amino acid substitutions.

The genetic group 6 evolved since 2011 and is further divided into three genetic subgroups 6A, 6B and 6C. A(H1N1)pdm09 viruses from this study (only two viruses; GHA/RV/219/2013 and GHA/RVp4/2014) were collected in the year 2013 and 2014, respectively. The two study viruses clustered closely with other viruses collected during the season 2013-2014 in Ghana, Cameroon, Belgium and South Africa. These viruses together fell into the genetic subgroup 6C represented by the reference strain A/Massachusetts/10/2013 and the clade-specific amino acid substitution V234I. The recent viruses circulating in Ghana from the season 2015 fell into genetic subgroup 6B clustering with the A/St. Petersburg/100/2011 reference strain and carried the specific amino acid substitutions S143G and A197T (Fig. 17a).

The phylogenetic analysis of the NA gene sequences of A(H1N1)pdm09 viruses revealed that the genetic groups were generally congruent with the HA phylogeny (Fig. 17b). The two

viruses from this study acquired the group specific amino acid substitution M19I. The strain A/Ghana/RVp4/2014 is further characterized by two additional amino acid substitutions I30T and Q313H (Fig. 17b). The amino acid change I30T may predict the addition of a potential O-glycosylation site at residue 30.

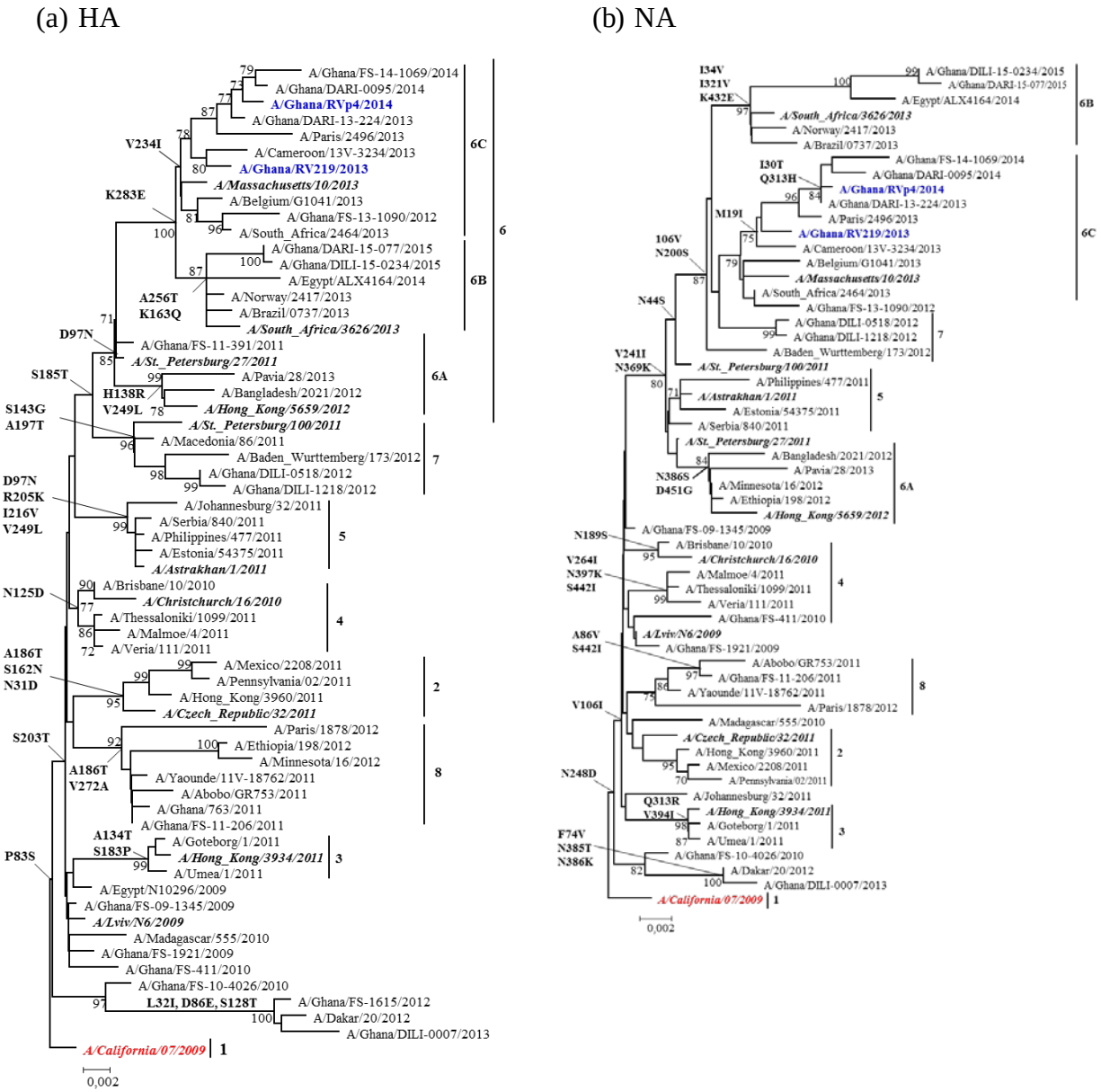


Fig 17: Phylogenetic analysis of HA and NA genes of influenza A(H1N1)pdm09 virus (a) HA gene (nt84-nt1713) and (b) NA gene (nt48-nt1452). The neighbor-joining tree was constructed with MEGA version 5.2 using the Kimura-2-parameter model with 1000 bootstrap replicates. Bootstrap values greater than 70% are displayed on branch nodes. Reference sequences of different genetic groups available in GISAID database are indicated by their accession numbers. The WHO recommended reference vaccine strain for is highlighted in red: Sequences from this study are highlighted in blue and designated by the geographic location (Ghana), patient number and year of collection. Deduced amino acid substitutions are related to A/California/07/2009. Clade-specific substitutions and fixed mutations are specified in bold on left side of node. Clades (1, 2, 3, 4, 5, 6A–6C, 7 and 8) are designated for both HA and NA trees.

3.9.1.3 Phylogenetic analysis of influenza B viruses

The phylogenetic analysis of the HA genes of influenza B viruses revealed two genetic clades of the B/Victoria-lineage (Fig. 18a). Currently circulating B viruses comprise two distinct lineages: B/Victoria and B/Yamagata. The two lineages co-circulated for several years allowing frequent reassortment among the viruses. The B/Victoria-lineage viruses identified in Ghana between 2009 and 2011 belonged to genetic clade 1B. The clade 1B is represented by B/Odessa/3886/2010 and B/Hong Kong/514/2009 reference strains with clade specific amino acid substitutions L58P and K275R.

Influenza B/Victoria-lineage viruses identified in Ghana from the season 2011 to 2015 including four viruses from this study fell into genetic clade 1A. The clade 1A is represented by the vaccine strain B/Brisbane/60/2008 and several reference strains including B/Paris/1762/2009, B/Malta/MV636714/2011, B/Formosa/V2367/2012, B/South Australia/81/2012 and B/Johannesburg/3964/2012. The recent viruses from the seasons 2013-2015 clustered more closely together. They acquired a common specific amino acid substitution K209N and fell into a distinct subgroup (Fig. 18a). Other viruses circulating during this time in some West African countries such as Cameroon, Togo and The Gambia as well belong to this subgroup.

Phylogenetic analysis of the NA gene sequences revealed that the B viruses from this study along with other reference B viruses from the 2013-2015 seasons clustered in a manner similar to their corresponding HA gene phylogeny (Fig. 18b). These viruses carried the specific amino acid substitutions D342N and M403V. Two viruses from this study were further characterized by additional amino acid substitutions G331E and G334N. The phylogenetic tree showed that the representative B viruses from Ghana of HA clade 1B clustered in NA clade 1A, suggestive of an intra-clade reassortment. Further, a reference strain from Ghana which belonged to the HA clade 1A clustered and in NA clade 3, indicative of inter-clade reassortment of (Fig. 18b).

(a) HA (b) NA

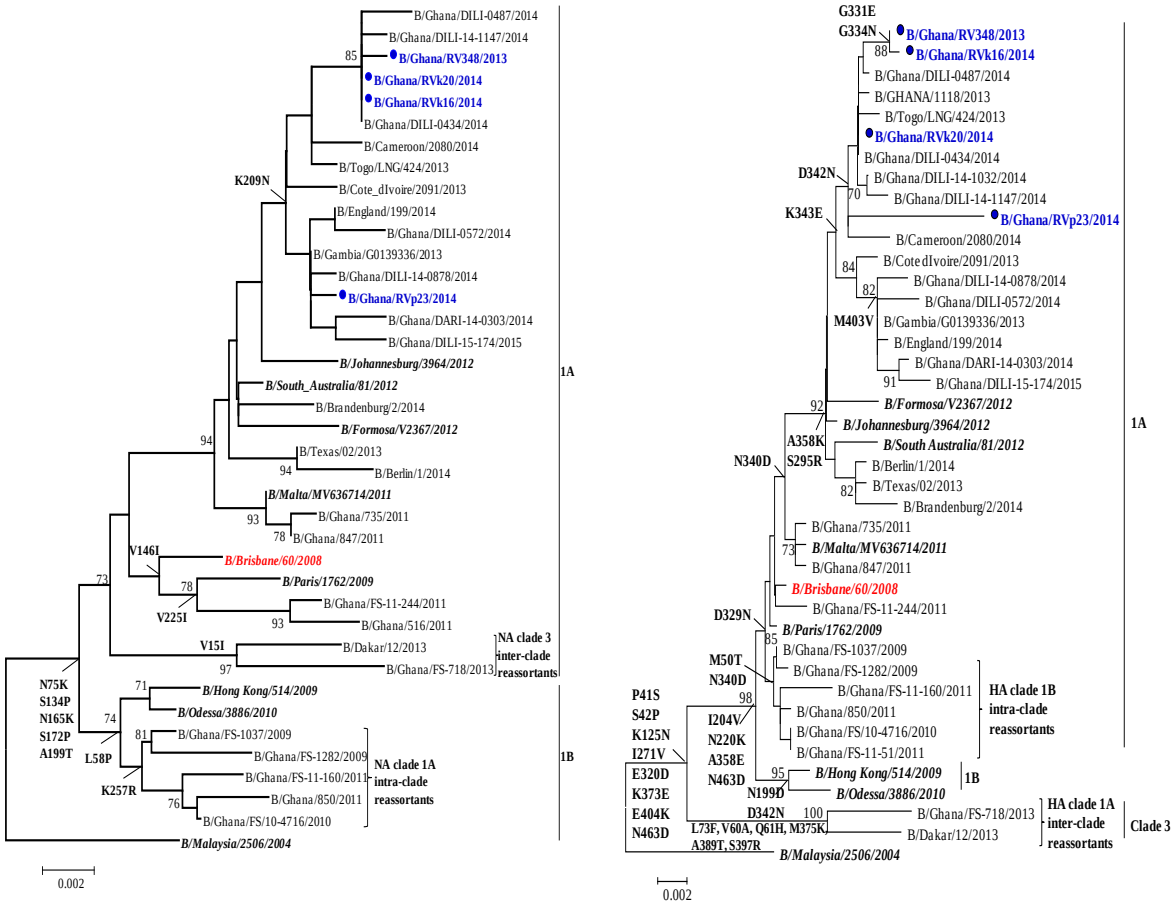


Fig. 18: Phylogenetic analysis of HA and NA genes of influenza B/Victoria-lineage virus (a) HA gene (nt28-nt1861) and (b) NA gene (nt20-nt1557). The neighbor-joining tree was constructed with MEGA version 5.2 using the Kimura-2-parameter model with 1000 bootstrap replicates. Bootstrap values greater than 70% are displayed on branch nodes. Reference sequences of different genetic groups available in GISAID database are indicated by their accession numbers. The WHO recommended reference vaccine strain is highlighted in red: Sequences from this study are highlighted in blue and designated by the geographic location (Ghana), patient number and year of collection. Deduced amino acid substitutions for B/Victoria-lineage viruses are related to A/Malaysia/2506/2004. Clade-specific substitutions and fixed mutations are indicated in bold on left side of node. Clades 1A and 1B representing the B/Victoria-lineage are indicated for both HA and NA trees. Clade 3 representing the B/Yamagata-lineage is indicated for the NA tree only.

3.9.2 Respiratory syncytial viruses

3.9.2.1 Phylogenetic analysis of RSV group A and B viruses

The second hypervariable region (VR2) of the G protein gene was sequenced for 46 RSV group A and 61 RSV group B viruses. The sequences were aligned in MEGA 5.2 and compared with reference sequences representing the different genotypes. RSV group A viruses belonged to 3 genotypes: ON1 (n = 40, 87%); NA1 (n = 5, 11%) and SAA2 (n = 1, 2%). RSV group B viruses belonged to 2 genotypes: BA9 (n = 60, 98%) and SAB4 (n = 1, 2%) (Fig. 19). Analysis of the circulation pattern of the different RSV genotypes showed that BA9 viruses predominantly co-

circulated with SAA2 during the season 2006, and with ON1 during the season 2013 (Table 19). However during the season 2014, ON1 viruses (96%) were the dominant genotypes co-circulating with NA1 (4%). The BA9 genotype virus was not present in the season 2014, neither was any other RSV group B genotype viruses found circulating in this year.

Table 19: Seasonal distribution of RSV genotypes

Season	Number of RSV-positives	Number (% of RSV-A viruses)			Number (% of RSV-B viruses)	
		ON1	NA1	SAA2	BA9	SAB4
2006	6	-	-	1 (17)	5 (83)	-
2013	72	13 (18)	4 (6)	-	54 (75)	1 (1)
2014	28	27 (96)	1 (4)	-	-	-

3.9.2.2 Intragenotype divergence of RSV genotypes

The nucleotide and deduced amino acid sequences of the second variable region of the RSV genotypes ON1, NA1 and BA9 viruses were compared within each genotype. The mean percentages of the nucleotide p-distance measure are shown in Table 20. The nucleotide divergence ranged between 0% and 14.7%, while the amino acid divergence ranged between 0% and 34.6%. Viruses belonging to the genotype NA1 showed lowest nucleotide distance and were more closely similar to each other, whereas a higher divergence was seen among the genotype BA9 viruses.

Table 20: Nucleotide and amino acid divergence of the RSV genotypes

RSV genotype	Number of viruses	Within group mean p-distance, % (range)	
		Nucleotide	Amino acid
BA9	60	2.9 (0-14.7)	5.7 (0-34.6)
ON1	40	1.5 (0-6.1)	2.7 0-11.8)
NA1	5	1.0 (0-1.9)	0.9 (0-2.4)

(a) RSV A (b) RSV B

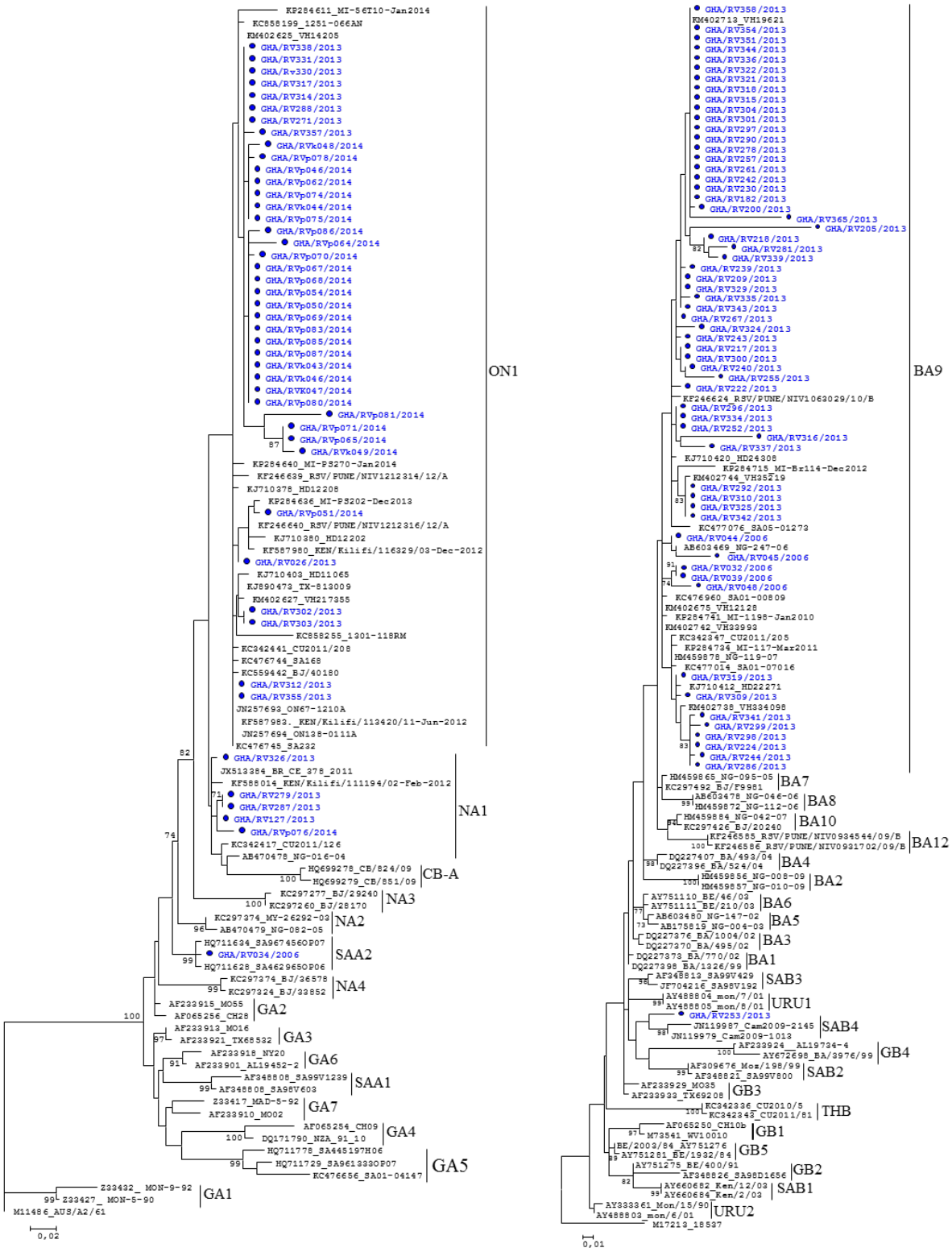


Fig. 19: Phylogenetic analysis of the VR2 region of G gene of RSV group A and B genotypes (a) Partial G gene sequences of RSV group A (nt634-nt969) and (b) RSV group B (nt652-nt975) viruses. The trees were constructed with MEGA version 5.2 using the Tamura-Nei and the Hasegawa-Kishino-Yano methods respectively, with 1,000 replicates. Reference sequences representing the different RSV genotypes were obtained from the GenBank and are indicated by their accession numbers. Sequences from this study are shown in bold blue color and designated by the geographic location (GHA), patient number and year of collection. The genotype clusters are indicated on the right side of figure. Only bootstrap values greater than 70% are displayed at the branch nodes.

3.9.2.3 Synonymous-to-nonsynonymous mutations of RSV genotypes

Using SNAP, the number of synonymous (ds) and nonsynonymous (dn) nucleotide substitution was estimated for the RSV genotypes obtained in the study. The ds/dn mutation ratio has been used as an indicator of selective pressure. A ds/dn ratio greater than one means a high abundance of synonymous (silent) mutations or negative selection, ds/dn ratio of equal to one means neutral mutation, and ds/dn ratio of less than one means positive selection. The analyses predicted an average ds/dn mutation ratio of 1.09 for genotype ON1, 1.08 for BA9, and 3.05 for NA1. These results indicated that genotype NA1 was under purifying or negative selection pressure in the variable region. A neutral selection pressure can be suggested for genotypes ON1 and BA9 for which an equal ratio of synonymous and nonsynonymous mutations have been observed.

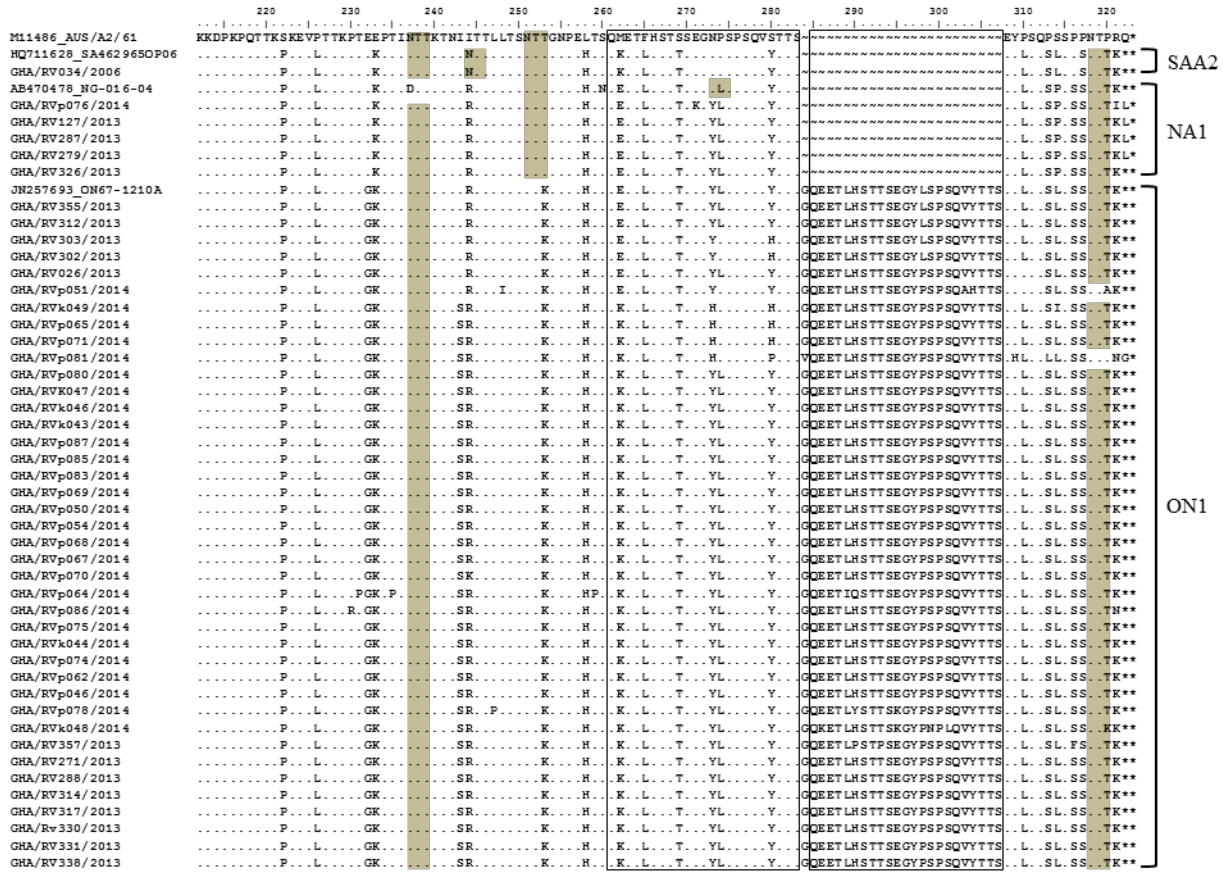
3.9.2.4 Analysis of the sequence of RSV group A viruses

RSV group A viruses showed genotype specific mutations when compared to the reference sequence RSV-A2 strain (Fig. 20a). The SAA2 genotype viruses acquired amino acid substitution I244N and ON1genotype viruses acquired E232G and T253K. Additional mutations specific to ON1 sequences from this study include I243S, M262K, N273H, S280H and L298P (L298P is a mutation with reference to the original 72-nt region prior to duplication). Viruses characterized by these additional amino acid changes belonged to observably separate subcluster in the phylogenetic tree (Fig. 19a). It was noted that a subcluster of three sequences (GHA/RVp071/2014, GHA/RVp065/2014, and GHA/RVk049/2014) was characterized by all five additional mutations. This resulted in a distinctively divergent subgroup supported by a high boot strap value of 87% in the phylogenetic tree.

The sequences for SAA2, NA1 and ON1 genotypes displayed different amino acids length due to alterations in their stop codon positions. The amino acid positions were 298 for SAA2, 299 for NA1 and 322 for ON1 genotypes. Stop codons were generally well conserved in all RSV-A genotypes. Except for one strain (GHA/RVp081/2014), the ON1 strains displayed an early G-gene stop codon at position 322. Again except for one strain (GHA/RV/326/2013), the NA1 sequences exhibited a stop codon at position 323. The NA1 viruses differed in their amino acid length when compared to the NA1 reference sequence NG-016-04. The NG-016-04 strain had an early stop codon at position 322 (Fig. 20a).

With the exception of GHA/RVp051/2014 and GHA/RVp081/2014 sequences, two potential N-glycosylation sites among ON1 viruses at positions 237 and 318 were observed. Moreover, these and two other putative N-glycosylation sites were identified in NA1 position 251 and SAA2 position 244 (Fig. 20a). Predicted O-glycosylation sites varied between the RSV-A genotypes; there were 37 to 41 residues in ON1 strains and 32 residues in NA1 and SAA2 genotypes.

(a) RSV A



To be continued on the next page

(b) RSV B

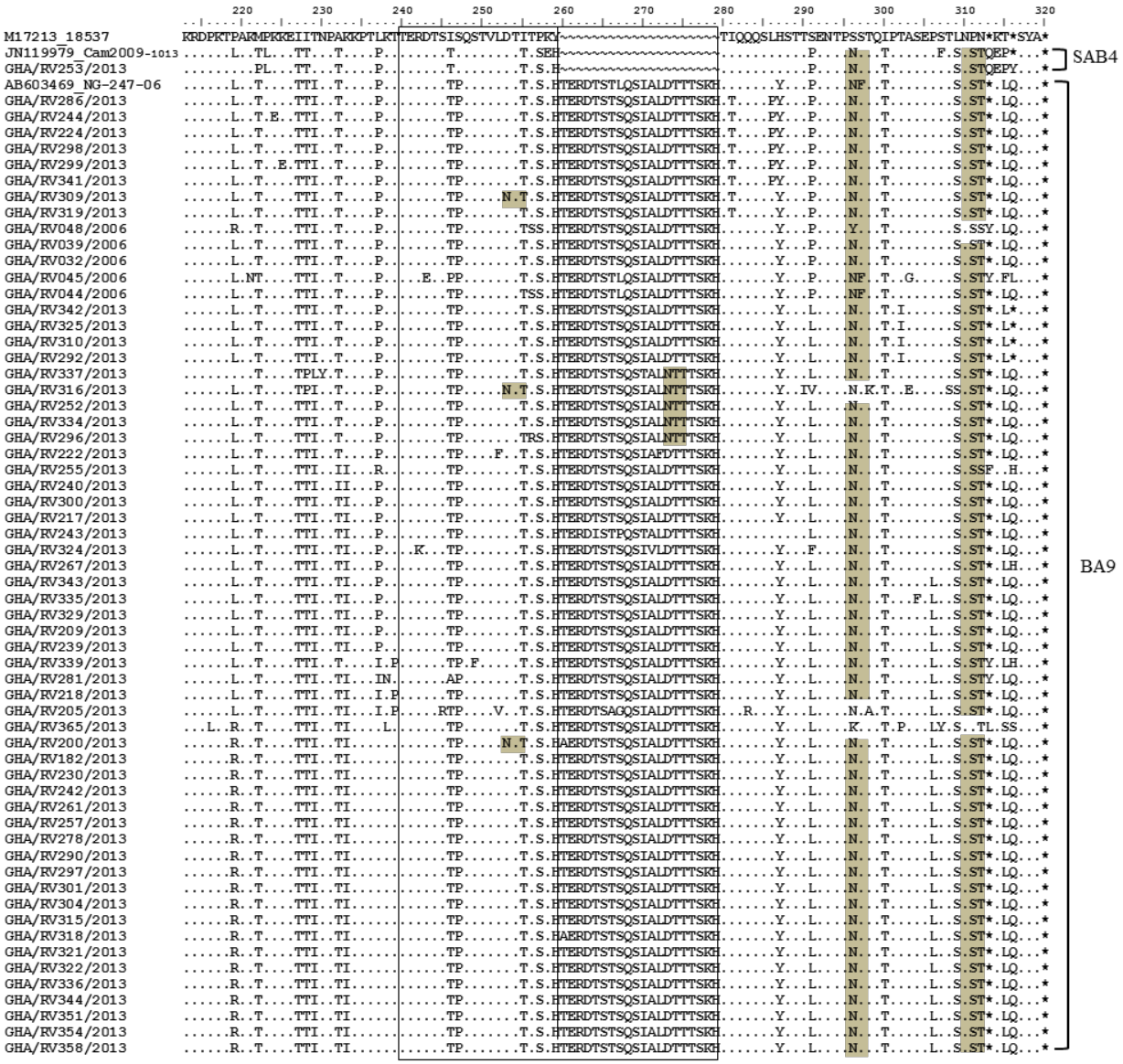


Fig. 20: Deduced amino acid alignment of the VR2 region of G gene of RSV group A and B viruses (a) G gene from RSV group A and (b) RSV group B viruses. Alignments are shown relative to the sequences of prototype strain A2, and genotype strains HQ711628_SA462965OP06-SAA2, AB470478_NG-016-04-NA1, and JN257693_ON67-1210A-ON1 for RSV-A; RSV-B prototype strain M17213_18537, and genotype strains JN119979_Cam2009-1013-SAB4 and AB603469_NG-247-06-BA9. Identical residues are indicated by dots. Stop codons are indicated by asterisks. Potential N-glycosylation sites (NXT/S, where X is not a proline) are indicated by gray shading. Rectangles indicate the two copies of amino acids duplicated regions. Sequences from this study are designated by the geographic location (GHA), patient number and year of collection.

3.9.2.5 Analysis of the sequence of RSV group B viruses

RSV group B sequences showed genotype specific mutations when compared to the reference sequence 18537 strain. The BA9 genotype viruses acquired T229I, S247P and H287Y (Fig. 20b). Additionally, BA9 viruses from this study showed different specific amino acid substitutions P219R, K233I, L237I, T239P, D273N (D273N is an amino acid change with

reference to the original 60-nt region prior to duplication), I281T, L286P, S291L, T302I and P306L. These amino acid changes defined several distinguished subgroups within the BA9 genotype cluster (Fig. 19b). For example, mutations L237I and T239P were unique to a subcluster comprising four viruses (GHA/RV205/2013, GHA/RV218/2013, GHA/RV281/2013, and GHA/RV339/2013); I281T and L286P were unique to a subcluster involving six viruses (GHA/RV224/2013, GHA/RV244/2013, GHA/RV286/2013, GHA/RV298/2013, GHA/RV299/2013, and GHA/RV341/2013); and T302I was unique to another subcluster comprising four viruses (GHA/RV292/2013, GHA/RV310/2013, GHA/RV325/2013, GHA/RV342/2013). It is worth noting that most of the divergent subgroups were supported by high bootstrap values ≥82%.

The alternating use of three different stop codon positions resulted in different amino acids length among the BA9 viruses. The stop codon displayed were at positions 313, 316 and 320. With the exception of four sequences, two potential N-glycosylation sites among BA9 viruses at positions 296 and 310 were observed. Furthermore, two other putative N-glycosylation sites were identified among three viruses (GHA/RV309/2013, GHA/RV316/2013, and GHA/RV200/2013) at position 253 and a group of five viruses (GHA/RV337/2013, GHA/RV316/2013, GHA/RV252/2013, GHA/RV334/2013, and GHA/RV296/2013) at position 273 (Fig. 20b). Predicted O-glycosylation sites varied between 40 and 45 residues.

3.9.3 Human metapneumovirus

3.9.3.1 Phylogenetic analysis of HMPV subgroups A and B

Partial amplification of the F protein gene and sequencing was carried out for 14 HMPV-positive samples. Phylogenetic analysis was performed using MEGA 5.2 software version and compared with reference sequences representing all the different genetic lineages. The phylogenetic analysis differentiated the sequences into the two main antigenic subgroups A and B (Fig. 21). The viruses identified in subgroup A further belonged to the genetic sub lineage A2a (2, 14%). The viruses in subgroup B further divided into the two genetic lineages, B1 (n = 1, 7%) and B2 (n = 11, 76%). No viruses from this study belonged to the genetic lineages A1 and genetic sub lineage A2b. The yearly distribution of HMPV showed that different lineages prevailed during the study period (Fig. 22). One virus identified in 2006 belonged to HMPV B1. All six HMPV identified in 2013 belonged to genetic lineage B2, whereas the year 2014 was characterized by the circulation of HMPV B2 (71%) and A2a (29%) viruses.

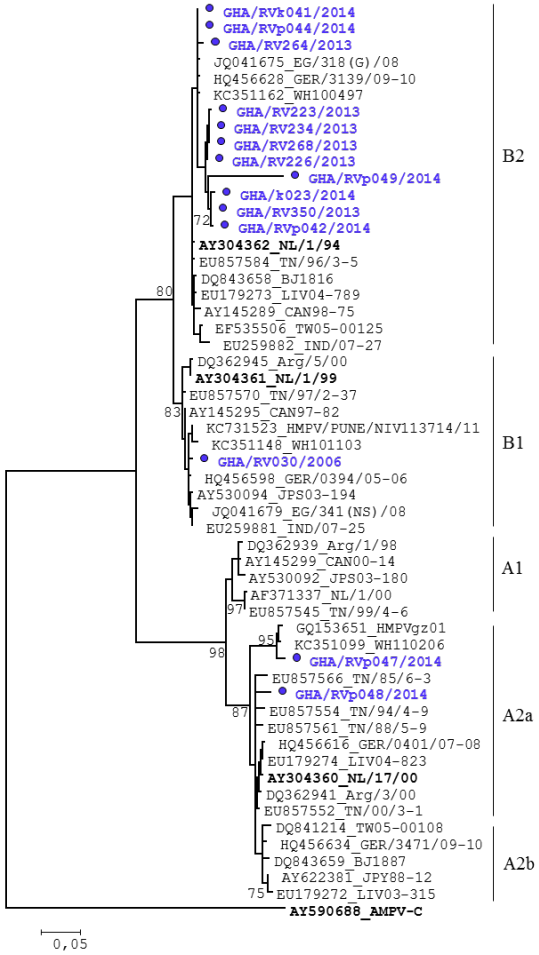


Fig. 21: Phylogenetic analysis of partial F gene fragments of HMPV. The tree was constructed in MEGA 5.2 using the HKY estimation with 1,000 replicates (nt597-nt1069). Avian metapneumovirus C (AMPV-C) was included as outgroup. Reference sequences representing the different HMPV genetic lineages were additionally included in the analysis, they are indicated by their Genbank accession numbers. Sequences from this study are shown in bold blue color and designated by the geographic location (GHA), patient number and year of collection. The lineages and sub-lineages are specified to the right of the figure. Only bootstrap values greater than 70% are displayed at the branch nodes.

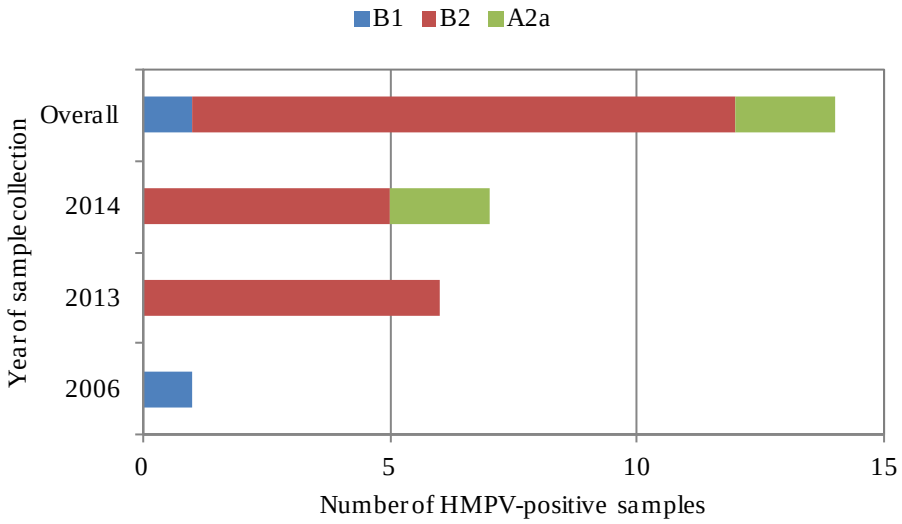


Fig. 22: Seasonal circulation of HMPV lineages and sub-lineages

3.9.3.2 Intragenotype divergence of HMPV lineages

Similarities within HMPV sequences from this study were higher at the amino acid level than at the nucleotide level (Table 21). The mean nucleotide p-distance within the B2 genetic lineage and A2a genetic sub-lineage was 2.9 and 5.5, respectively, while mean nucleotide p-distances at the amino acid level within the B2 genetic lineage was 2.5.

Table 21: Nucleotide and amino acid divergence of the HMPV lineages

		Within group mean distance (%)	
HMPV genetic lineage	**Number of viruses**	**Nucleotide level**	**Amino acid level**
B2	11	2.9 (0-9.7)	2.5 (0-11.6)
A2a	2	5.5 (0-5.5)	0 (0)

3.9.3.3

3.9.3.4 Synonymous-to-nonsynonymous mutations of HMPV lineages

Using SNAP, the number of synonymous (ds) and nonsynonymous (dn) nucleotide substitution was estimated for the HMPV genotypes obtained in the study. The ds/dn mutation ratio has been used as an indicator of selective pressure with a ds/dn greater than one signifying purifying or negative selection, ds/dn equals one signifying neutral selection, and ds/dn less than one signifying positive selection. The analyses predict an average ds/dn mutation ratio of 5.9 for genotype B2 and 6.7 for genotype A2a indicating a purifying or negative selection pressure on the circulating HMPV strains.

3.9.3.5 Deduced amino acid analysis of HMPV lineages

The nucleotide sequences of HMPV showed lineage specific mutations when compared with the reference strain AMPV-C. The amino acid substitutions N233Y, V286I and Q312K were specific for HMPV lineages B1 and B2 viruses, whereas the amino acid substitutions D296K and E348K were specific to HMPV sub-lineage A2a viruses (Fig. 23). Further unique amino acid mutations T223N and D280N were acquired by all the HMPV B2 sequences from this study. Moreover, these HMPV B2 viruses formed a separate subcluster with relation to the HMPV B2 reference strain NL/1/94 in the phylogenetic tree analysis (Fig. 21).

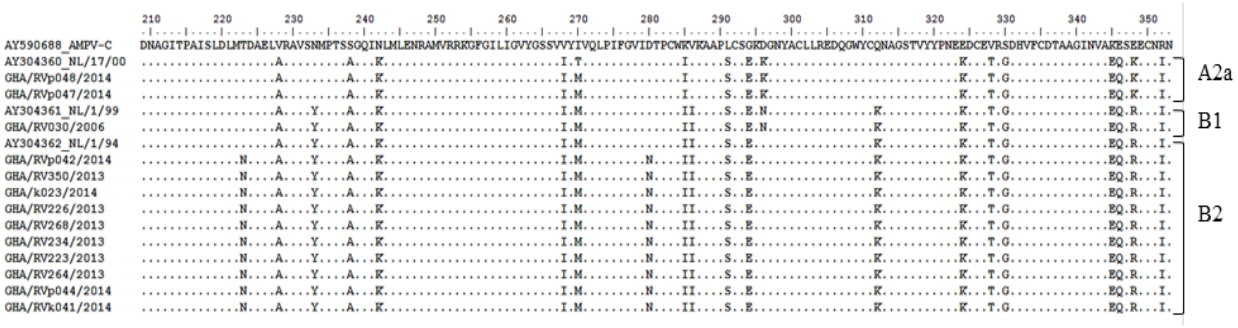


Fig. 23: Deduced amino acid alignment of partial F gene of HMPV. Alignments are shown relative to the out-group reference sequence of strain AMPV-C. Lineage specific reference sequences are represented by NL/17/00 for A2a, NL/1/99 for B1 and NL/1/94 for B2. Identical amino acid residues are indicated by dots. Sequences from this study are designated by the geographic location (GHA), patient number and year of collection.

3.9.4 Human Adenoviruses

3.9.4.1 Phylogenetic analysis of HAdV types

The hexon and fiber genes of the 64 HAdV-positive samples from this study were amplified and sequenced for the genetic characterization of the HAdV. Phylogenetic analysis of the hexon gene sequences was performed for 34 HAdV, and phylogenetic analysis of the fiber gene sequences was performed for 25 HAdV using MEGA 6.0 software version. The sequences were compared with reference sequences representing the different HAdV species and types.

The analysis revealed that 13 different HAdV types circulated during the study period as follows: HAdV type 1, 2, 3, 4, 5, 6, 7, 24, 40, 41, 57, 61 and 64 (Fig. 24). The number of samples determined for each type is also shown (Table 22). Among species C, HAdV-C1 was most frequently identified in 11 of 18 samples, and among species B, HAdV-B3 was most frequent in 8 of 12 samples. It was noted that four viruses belonging to the HAdV-B7 in the hexon gene phylogeny clustered with HAdV-B3 viruses in the fiber gene phylogeny. This is suggestive of an intra-species recombination of these viruses.

Table 22: Distribution of HAdV types among different species

HAdV species	Total number of viruses sequenced	HAdV type (number of cases)
A	1	A61 (1)
B	12	B3 (8), B7 (4)
C	18	C1 (11), C2 (1), C5 (2), C6 (3), C57 (1)
D	3	D24 (1), D64 (2)
E	2	E4 (2)
F	4	F40 (2), F41 (2)

(a) Hexon (b) Fiber

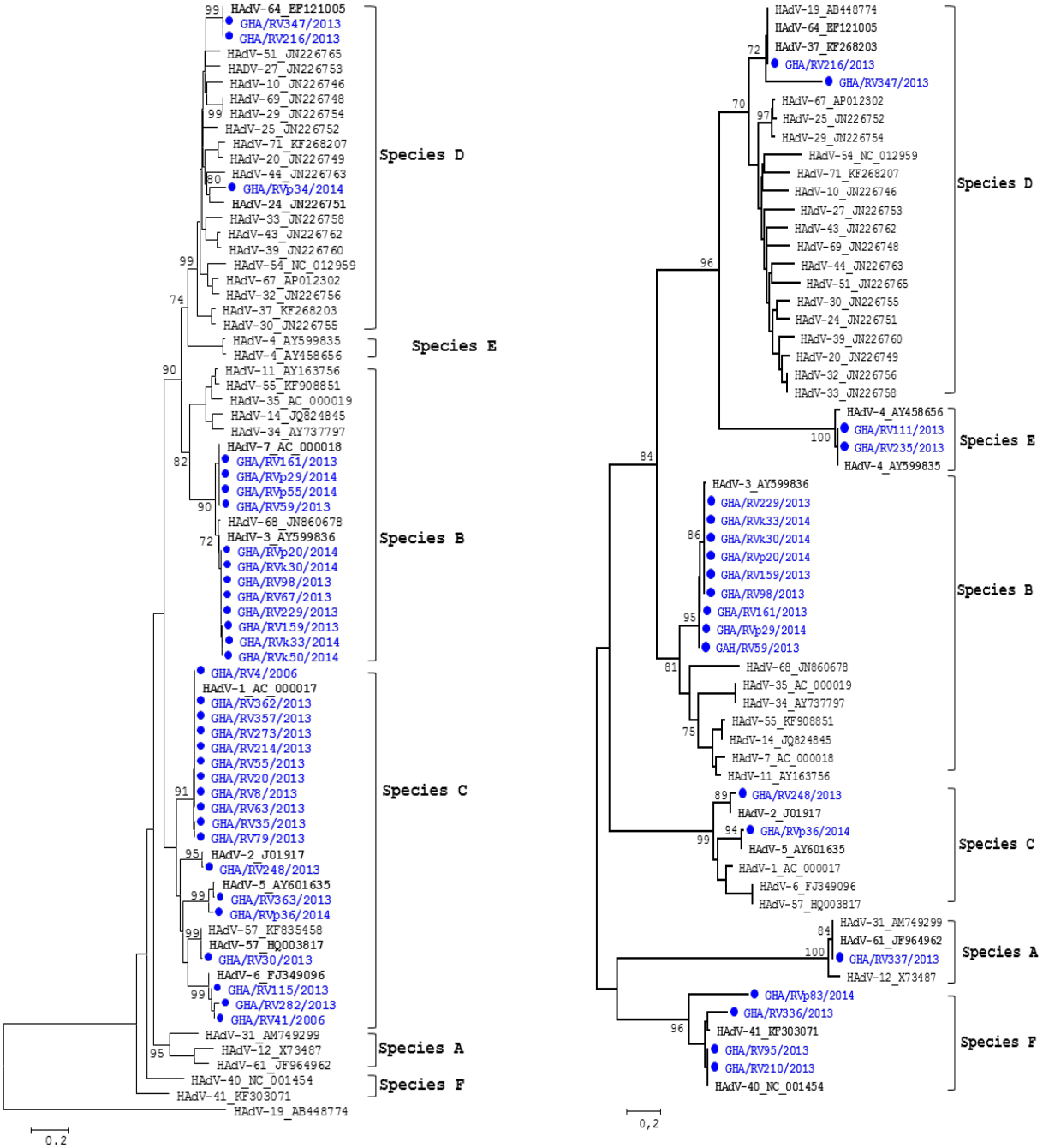


Fig. 24: Phylogenetic analyses of HAdV based on the partial hexon and fiber genes (a) Hexon gene (nt211-nt1745) and (b) fiber gene (nt1652-nt2614) sequences of HAdV types. The MEGA 6 software package was used to generate the phylogenetic trees applying the Neighbor joining method (Maximum composite likelihood) and 1000 replicates for bootstrap analysis. Reference sequences are indicated by their accession numbers. Sequences from this study are highlighted in blue color and designated by the geographic location (GHA), patient number and year of collection. HAdV species are specified on the right side of each tree.

3.9.5 Human Rhinoviruses

3.9.5.1 Phylogenetic analysis of RV species

The VP4/VP2 coding region was amplified and sequenced for the 103 RV-positive samples detected during this study in order to identify the infecting genetic types. Phylogenetic analysis could be performed on 79 RV-positive sequences and compared first with reference sequences of the three RV species for representation into RV species A, B and C. Among the sequenced samples, 36 (46%) were classified as RV species A, 5 (6%) as RV species B and 38 (48%) as RV species C species (Fig. 25.). The distribution of RV species according to the season of circulation is shown in (Table 23). RV species B species predominated in 2006 and 2013; RV species C prevailed in 2013.

3.9.5.2 Genetically assigned RV types

In order to evaluate the genetic diversity within each of the RV species A, B, and C, separate phylogenetic trees were constructed. Further, the criteria for classification of RV species A, B and C presented by McIntyre et al [171] was used to differentiate the viruses into genotypically assigned types. A divergence threshold of 10.5, 9.5 and 10.5% was proposed for identifying different RV-A, -B and -C types respectively. Based on these criteria, a large number of different circulating RV types were determined. There were 16 types of RV species A, four types of RV species B and 20 types of RV species C (Fig. 26). Among RV species A, RV-A12 was the predominant type, followed by RV-A16, -A49, and -A101 (Table 22). Among RV species C, RV-C2 was the most dominant type, followed by RV-C 23. Seven variants of novel RV types were identified among RV species A and C. These variants 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 RV-Apat_a (3 viruses), RV-Apat_b (2 viruses), RV-Cpat_a (1 virus) and RV-Cpat_b (1 virus). These viruses await sequence data from their VP1 gene to confirm their assignment as putative new types. It was noted that there was rapid turnover of the RV types. Of the 40 different RV types identified, only one type (RV-A12) was found to have circulated in all the three seasons of study, and three (RV-Apat-a, RV-C23, and RV-C39) were found to have circulated in two seasons.

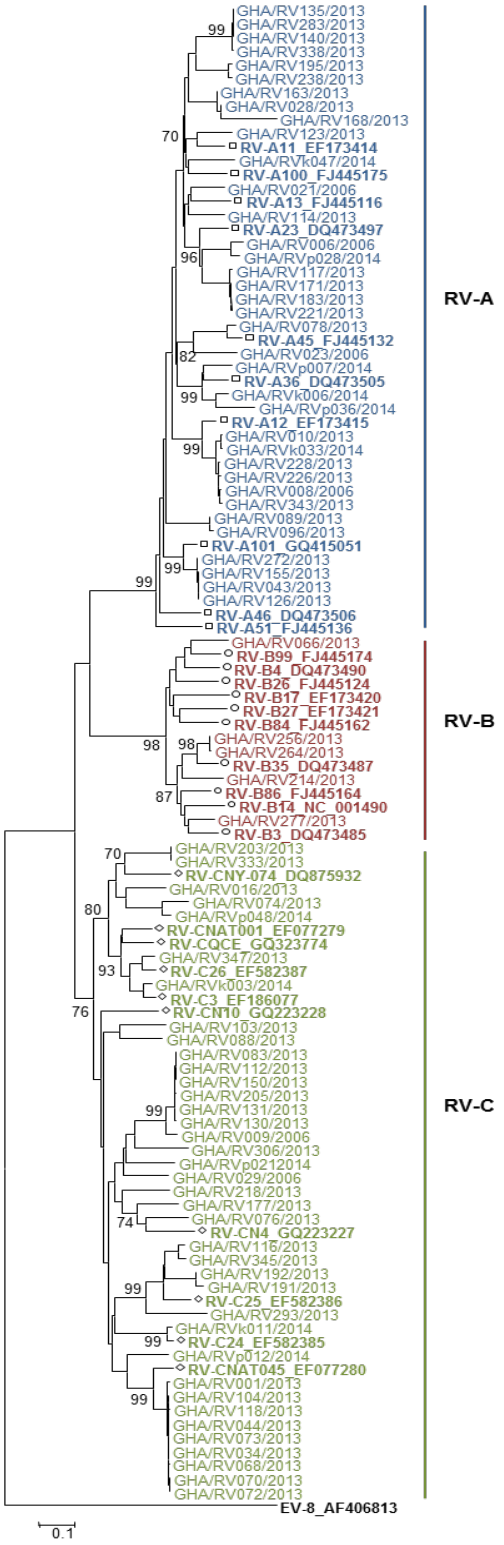


Fig. 25: Phylogenetic analysis of the VP4/VP2 region of RV species. Differentiation of RV species A, B and C viruses detected in this study was based on sequences in the VP4/VP2 region, (nt616-nt1004). For the phylogeny, neighbor-joining trees were constructed by using maximum-composite-likelihood method. Data were bootstrap resembled 100 times to assess the robustness of the branches; values of 70% or greater are shown. Sequences belonging to RV-A are colored blue, RV-B are red and RV-C are green. Reference strains of species A, B, and C available in GenBank are indicated by their accession numbers and are boldface. Sequences from this study are designated by the geographic location (GHA), patient number and year of collection.

(a) RV-A (b) RV-B (c) RV-C

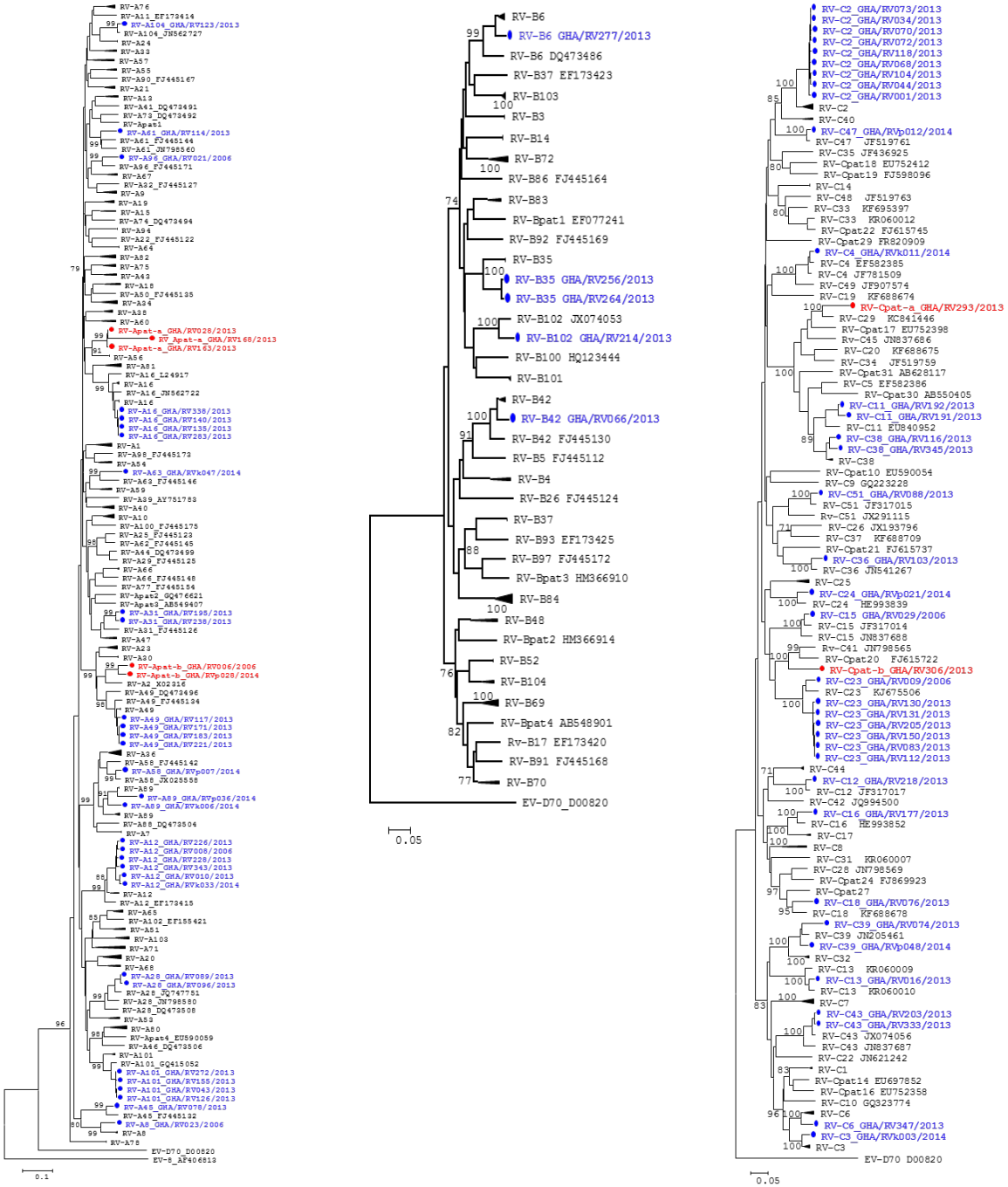


Fig. 26: Phylogenetic analysis of RV types based on the VP4/VP2 gene regions (a) RV-A, (b) RV-B and RV C; (nt616-1004). The tree was constructed by a neighbor-joining method from 1000 bootstrap resembled sequence alignments of maximum composite likelihood distances. Multiple reference sequences of the same type are shown in compressed triangles. Other references are indicated by their accession numbers. Sequences of RV provisionally assigned types (PATs) from this study are highlighted in bold blue and red colors respectively, and designated by the geographic location (GHA), patient number and year of collection.

Table 23: RV types detected among study patients

RV species and type	Total number of viruses	Number of viruses indicated per year		
		2006	2013	2014
Species A (n = 16 types)				
A 8	1	1		
A 12	6	1	4	1
A 16	4		4	
A 28	2		2	
A 31	2		2	
A 45	1		1	
A 49	4		4	
A 58	1			1
A 61	1		1	
A 63	1			1
A 89	2			2
A 96	1	1		
A 101	4		4	
A 104	1		1	
Apat-a	3		3	
Apat-b	2	1		1
Subtotal	**36**	**4**	**26**	**6**
Species B (n = 4 types)				
B 6	1		1	
B 35	2		2	
B 42	1		1	
B 102	1		1	
Subtotal	**5**	**0**	**5**	**0**
Species C (n = 20 types)				
C 2	9		9	
C 3	1			1
C 4	1			1
C 6	1		1	
C 11	2		2	
C 12	1		1	
C 13	1		1	
C15	1	1		
C 16	1		1	
C 18	1		1	
C 23	7	1	6	
C 24	1			1
C 36	1		1	
C38	2		2	
C39	2		1	1
C 43	2		2	
C47	1			1
C 51	1		1	
Cpat-a	1		1	
Cpat-b	1		1	
Subtotal	**38**	**2**	**31**	**5**
Total	**79**	**6**	**62**	**11**

4 Discussion

4.1 Respiratory viruses as cause of ALRI

ALRI are a leading cause of childhood morbidity and mortality in developing countries [1, 2]. In Ghana, ALRI have a major impact on the disease burden among children [19]. However the etiological agents of ALRI are rarely sought and/or limited to few pathogens. This study describes for the first time, a comprehensive viral etiology of ALRI among children 0-5years old in Ghana. Studies elsewhere have demonstrated that viruses are responsible for a large proportion of ALRI in children, but antibiotics are often prescribed for viral illnesses [5, 30]. Moreover, the viral etiologies of ALRI in children may differ according to age of a child, clinical presentation, season, and geographical setting [131, 241]; and different pathogens may show similar symptoms [28, 33]. Furthermore, pathogens such as influenza viruses, RSV, and other respiratory viruses are continually changing their antigenicity and challenging our health care systems. Additionally, underlying diseases like malaria and HIV infection may alter the pattern of ALRI. Hence, establishing the cause of ALRI in patients and the relative contribution of individual viruses have the potential to reduce overall antibiotic use, and to improve the targeted use of antibiotics and antiviral drugs. In addition, identification and characterization of viral infections would generate epidemiological data that may be useful in the design of vaccines for respiratory viruses such as RSV, as well as effective implementation of recommended vaccines for viruses such as influenza viruses.

In comparison to previous ALRI studies from Ghana [21, 22], the present study surveyed a larger diversity of respiratory viruses and a larger number of patients. Altogether, 552 patients from two hospitals in Accra were prospectively enrolled during the three years; from 2006 (47), 2013 (365), and 2014 (150). Eligible patients were uniformly recruited and respiratory specimens were collected at the time of admission. Obviously most samples were obtained in 2013 and there were fewer samples in 2006 and 2014. The voluntary participation of patients may explain the uneven yearly proportions of respiratory samples collected over the study period. Additionally, some polyclinics were upgraded and no longer referred patients to the study hospitals resulting in lower sample numbers in 2014.

The majority (53%) of these patients were below one year of age. Besides, the number of patients and moreover the risk of ALRI decreased with increasing age, for example 24% in 1-2 years old patients and 14% in 2-3 years old patients. Likewise in a study of 759 Kenyan infants

and children, the incidence of hospital admission with severe pneumonia ranged from 4.8% in the first year of life to 0.1% among older children [14]. In different 3-year prospective studies from Cambodia [241] and Niger [227], the majority of patients with ALRI 51% and 56%, respectively, occurred in infants less than 12 months, suggesting that age is a factor in ALRI. Moreover, the infant immune system is thought to be immature and in combination with small body size and small airways may contribute to the development of severe ALRI [7].

All patients were investigated by real-time PCR for 16 common respiratory viruses. These were influenza A and B viruses, RSV A and B, HMPV, HAdV, RV, HPIV1-4, HCoV-229E, -OC43, -NL63 and HKU1, and HBoV. Overall, 73% of patients were positive for one or more respiratory viruses. A comparable viral prevalence was estimated in many other studies from tropical countries. For example, nasopharyngeal aspirates from children aged below three years with ARI in Burkina Faso were screened for 10 viruses and suggested a viral etiology in 73.2% of patients [226]. In another study from Brazil which tested for 13 viruses, at least one virus was detected in 85% of patients less than three years old with lower respiratory tract infections (LRTI) [242]. However, in studies from Egypt [243] and South Africa [244] a slightly lower viral prevalence of 59.9% and 62.9%, respectively was reported.
The prevalence of viral respiratory infections may vary in different studies for several reasons; nevertheless, the scope of investigated pathogens may play a significant role in the viral positivity rate. Generally the role of respiratory viruses in ALRI has become increasing important and their contribution to respiratory disease cannot be over emphasized [28].

It is clearly reflected in the present comprehensive study of patients with ALRI that, RSV was the most frequently detected virus (23%), followed by RV (19%). Likewise in reports from Kenya [14], Brazil [242] and Egypt [243], RSV was the most commonly detected virus in 34%, 54% and 23% patients, respectively. Contrary, RV was the most common pathogen identified followed by RSV in other studies from the tropics. The RV prevalence from this study was lower than rates reported from Burkina Faso (59%) [226], South Africa (39%) [244], Cambodia (34%) [241] and Mozambique (26%) [245], but higher than in reports from Cameroon (17.9%) [16] and Senegal (14.6%) [18]. A study from North-East Brazil also reported on a prevalence of RV of 19% among ALRI [246].

In this study, HPIV and HAdV were equally prevalent among patients. The prevalence of 12% for HPIV was the highest so far when compared with earlier reports from Ghana (3%) [22],

Egypt (5%) [243], Cambodia (8%) [241], and Brazil (6.5%) [242]. HAdV was detected in 12% of patients which falls within the 3% and 18% detection range reported by some studies in Africa [14, 22, 243]. Influenza virus was one of the least detected pathogen (6%), besides HCoV (6%), and HMPV (3%) in this study. In line with other reports, influenza virus has been identified in quite comparable proportions of 0.8%-6% among children with ALRI [13, 22, 241, 247, 248], suggesting that influenza virus plays a minor role in this disease etiology. However, among children with ILI, a higher prevalence of 12%-34% has been observed in Ghana and elsewhere in Africa [238, 248-250]. HCoV has been reported as comparatively uncommon cause of ALRI among 1.4%-5.4% hospitalized patients [16, 241, 251, 252]. Contradictorily, in a study among outpatient children with ARI, HCoV was identified in 12.5%. The prevalence of HMPV from this study is slightly lower than the 4%-12% detection range reported by other tropical countries [149, 150, 245].

When considering ALRI in children, RSV is consistently the leading cause especially for young children. The detection rates of the other viruses differ, probably due to the occurrence of epidemics, different methods of detection, different study populations and geographical locations, and selection criteria. The most frequently reported viruses in children less than 5 years of age in most tropical areas were RSV, RV, HPIV3, HAdV and influenza virus.

Primarily, infants and children were often affected by respiratory viruses. Moreover among patients of this study, RSV, HBoV and influenza virus were significantly age dependent. RSV was significantly associated with the infant group (33%), whereas HBoV (21%) was most prevalent among the toddler group (1-3 years) and influenza virus (22%) among the preschool group (4-5 years). Other viruses had no significant association to age. Moreover, RSV was less detected in toddlers (14.5%) and preschool children (6%) implying that the risk of RSV infection decreased with increasing age. Conversely, the risk of an influenza virus infection increased with age; only 3% of infants were affected. Relatively RSV was present in the majority of infants (192/453, 42%) from Kenya [14] and in 51% among infants younger than three months in Germany [66]. An influenza surveillance report from Ghana demonstrated a similar age relation as observed for influenza virus from patients of the present study, whereby the proportion of ILI cases positive for influenza virus was 11% among infants and 31% among children aged 5-10 years [238]. Other studies have also demonstrated that older children experience high attack rates of influenza virus and therefore play an important role in community-wide transmission of the virus [13, 253]. HBoV was associated with toddlers (1-3 year old). In a comparable study from South Africa, all HBoV patients were younger than two

years of age [218]. Similarly in an earlier study from Germany, HBoV was significantly associated with LRTI in 1-3 year old children. ALRI are common in children, and they decrease with increasing age. This is especially true of RSV infection, where infants form the majority of those infected.

ALRI have often been associated with multiple viral infections. The samples of this study were investigated for a broad subset of respiratory viruses, making it possible to detect coinfections. Coinfections were detected in 24% of the samples. A viral coinfection rate between 6% and 14% has been reported by comparable studies from tropical countries [226, 241]. Additionally, from a study in Brazil where a high viral coinfection rate 56% was observed, samples were collected only during the months of greatest prevalence for acute pediatric respiratory viral illnesses [242].

In this study RV, HBoV and HAdV were the most commonly identified viruses in double and triple viral infections. RSV and HMPV were additionally reported to be involved in coinfections. Frequently identified viral combinations by other studies included RV-RSV [226]; RV-RSV/RV-HBoV [241]; RSV-HMPV/RV-HMPV-IV [242]; and RSV-IV/HMPV-HAdV-HBoV [254] which are parallel with the observations from this study. Presently, the clinical significance of viral coinfection remains ambiguous due to conflicting reports. In a study from Brazil, RSV and RV coinfections were associated with increased length of hospital stay and oxygen use especially for infants younger than six months [242]. Contrary, no significant relation to disease severity was observed for viral coinfections in other investigations from Cambodia [241] and Mexico [254]. Moreover, recurrent infection, viral persistence and prolonged nasopharyngeal shedding of RV, HBoV and HAdV have been reported in both symptomatic and asymptomatic patients [74, 151, 255], therefore their pathogenic role in coinfections is unclear.

Especially for HBoV, their role in disease etiology is unclear. The 14% prevalence rate of HBoV in this study was higher than the 1.5%-11% in previous reports from some tropical countries [199, 217, 218]; except in the report from Kenya were 16.8% was recorded [256]. Nevertheless, 58% of the HBoV detected in this study were involved in coinfections. Not many studies have looked at HBoV infections from the tropics, however, between 14% and 44% coinfection rates have been reported [199, 217, 218, 256]. From the temperate regions, a high coinfection rate of up to 83% in respiratory samples from HBoV-positive patients has been described [198]. In a prospective study of infants and toddlers attending daycare centers in the

USA, 72% (76/106) HBoV-positive cases had coinfections [211]. Additionally, illness due to HBoV alone (with no coinfecting viruses) was not associated with the presence of respiratory illness symptoms or severity of illness. Nonetheless one case of HBoV infection with severe respiratory illness was also infected with RSV, HCoV, RV, and HAdV [211]. Other studies among asymptomatic subjects have shown that HBoV may exist in the respiratory tracts as a bystander without causality to the current symptoms or illness [212, 213].

4.2 Circulation of respiratory viruses

The climate of Ghana is tropical and generally characterized by a wet or rainy season from April to October, and a dry hot season from November to March [240]. About two third of the annual rainfall occurs during the wet season, with higher humidity levels and comparatively low temperatures. RSV predominantly circulated during the rainy season with a higher seasonal activity in October. This trend of RSV circulation was observed in the majority of previous investigations from sub-Saharan Africa, including Ghana [22], Senegal [18] and Cameroon [16] where the peak infection rate of RSV occurrence coincided with the rainy period in October. Reports from other tropical regions such as Cambodia [241] and Brazil [242] also showed the seasonality of RSV corresponded with the rainy season. Though, few studies from the tropics demonstrate RSV correlation with the dry season [21, 226, 227].

In temperate regions, RSV infections peak in the winter. There reasons that have been suggested are crowding of susceptible individuals indoors during winter [257]; and the cooling of the nasal passages with concomitant decrease in respiratory defense may contribute to its strong seasonality [258]. In Africa however, data on RSV circulation are limited and the reasons for the strong seasonality are unclear. At best it may be speculated that during the rainy season, children tend to be kept indoors and the resultant crowding may account for the increased incidence of RSV during this period. Another suggestion 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 under high temperatures [259], which may explain the relationship with cooler weather.

HAdV, HPIV, RV and HBoV circulated concurrently throughout the year, but peaked in February and may explain their extensive coinfections among patients. Most of these group of viruses that have been studied elsewhere were either endemic throughout the year, detected sporadically or associated with epidemics. A 4-year study from rural Thailand found HPIV

seasonal peaks between January and April [188]. However, HAdV, HPIV, RV and HBoV did not show any marked seasonality but rather a year-round and/or sporadic circulation in studies from Brazil [260] and Madagascar [261]. It is therefore difficult to draw conclusions about their distribution and seasonal patterns.

The seasonality of HMPV, IV and HCoV could not be deduced from the few positive patient samples found in each month. However in other comparative studies from Cambodia [147] and Brazil [150], HMPV usually circulates during the rainy season, as in the case of RSV. Influenza virus surveillance among ILI/SARI in Ghana demonstrated a year-round circulation of influenza virus, with a positive correlation of the timing of case peaks and rainfall [238, 239]. From Senegal [18] and Cameroon [16], the influenza virus activity was higher and coincided with a high RSV occurrence in October during the rainy season and only few cases could be detected during the dry season. In temperate countries, influenza epidemics are more common in the winter [262]. In general, the respiratory viruses identified here showed a circulation pattern specific to their tropical climate, with RSV circulating in the rainy season and HAdV, HPIV, RV and HBoV mainly in the dry season.

Patients with ALRI were recruited all through the year with the highest collection numbers in February and October (Fig. 27). It seems that there is a three-phase occurrence of ALRI; one in the dry season (peak in February) and two others in the rainy season (peaks in July and October). The burden of ALRI may be influenced by the circulation of multiple viruses. Meaning that, HAdV, HPIV, RV and HBoV induce ALRI in the dry season and mainly RSV induce ALRI in the rainy season. In addition, weather variability such as rainfall and humidity not only influences the circulation of viruses [131, 263] but is also associated with particularly high levels of ALRI [264, 265].

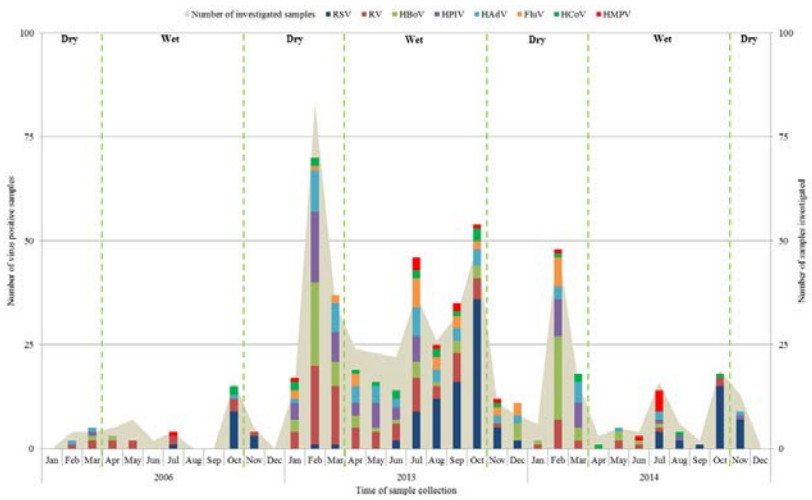


Fig. 27: Seasonal distribution of virus positive samples from children with ALRI

4.2.1 Distribution and circulation of HPIV types

HPIV were detected in 65 of 552 respiratory samples. Differentiation of the HPIV positive samples showed that HPIV3 was predominating with 51%. Remarkably, HPIV1 and HPIV4 were found in approximately equal proportions with a detection rate of 22% and 23%, respectively. HPIV2 was less detected (5%). The majority of studies have focused mainly on HPIV1-3 infections probably because of their high positivity rate and morbidity among children [185, 247, 261, 265-267]. Predominance of HPIV3 was observed by the majority of HPIV studies. Furthermore, in an Italian study of hospitalized children with ARI, HPIV3 was the only type identified in 7.5% of HPIV-positive cases [268]. On the contrary, HPIV1 was predominant in oropharyngeal swabs obtained from individuals with ILI from 10 Latin-American countries [188]. The epidemiology of HPIV4 has been less described due to its low detection of less than 3% in most previous studies [50, 188, 261, 269]. Nevertheless HPIV1-4 was retrospectively analyzed in the USA [51]. There, HPIV4 predominated over the other three types with a detection rate of 34% and interestingly, all the HPIV types co-circulated during the study.

In this study, the proportion of the four HPIV types in the season 2013 was comparable to the season 2014, with each HPIV3 predominating. Likewise in long-term studies from Brazil [185], Thailand [267] and Malaysia [265], HPIV1 and HPIV3 often co-circulated in each season, and the predominance of HPIV3 was demonstrated among the circulating HPIV types. In the USA, HPIV3 can occur throughout the year with increased activity in spring and summer, while HPIV1 and HPIV4 had year-round prevalence with peaks in the autumn of odd-numbered years [51, 187]. Such a circulation pattern can hardly be described in the present study since only two consecutive seasons are monitored. But by trend, there is a higher and prolonged circulation of HPIV3 from January-July in 2013 compared to a less circulation from January-March in 2014. It seems that HPIV1 follows HPIV3 circulation in 2013, but this is speculative. Indeed only comprehensive surveillance extended over a couple of years will provide the basic data needed to definitely define HPIV seasonality in Ghana.

4.2.2 Distribution and circulation of HCoV species

Within this study, 25 HCoV-positive samples were differentiated into the corresponding species OC43, 229E, NL63 and HKU1. There was dominance of the OC43 and 229E species accounting for 36% and 32% infections, respectively. The other HCoV species were less frequently detected. A predominance of OC43 and NL63 has been more frequently reported

among HCoV infections by other studies from Kenya [194] and Thailand [235]. Furthermore, from South Africa [195] and Brazil [80] only large numbers of NL63 species were detected. There is no clear explanation for the higher frequency of infection for OC43 and NL63 compared to HKU1 and 229E among children. However, a study from the Netherlands hypothesized that, an infection by NL63 elicits neutralizing antibodies directed to the NL63-spike protein that might also protect, or partially protect, against 229E infection, whereas this relationship may not be reciprocated, thus providing a greater likelihood of NL63 infection than 229E. The same was speculated for OC43 for which neutralizing antibodies may protect against HKU1 infection, but not vice versa [192]. There are no indications that infection by one of the HCoV is more pathogenic than others [79]. Nevertheless, NL63 has been associated with croup [81].

While there were yearly variations in the prevalence of HCoV infections throughout this study, the data also revealed that during 2013, there was co-circulation of four HCoV species which particularly coincided with a high rate (68%) of the HCoV infections identified. However, only one sample was infected with 229E and HKU1. Few studies from both temperate and tropical regions have identified co-circulation of all four HCoV species [80, 191, 194, 235], and coinfections between the species are not commonly reported [79, 191, 196]. For example, in a prospective study of children with ALRI in the USA, two patients had a coinfection with HKU1 and OC43, and one patient with NL63 and 229E [270]. The infrequent coinfections between HCoV species may be linked to possible antibody cross-reactivity among members of the same group [192].

4.3 Association of respiratory virus with clinical manifestation of ALRI

Children with ALRI were investigated for 16 respiratory viruses. Moreover, ALRI was defined as fast breathing in addition to cough, nasal discharge or fever. Under these circumstances, diagnosis of bronchopneumonia and bronchiolitis was significantly associated with RSV as well as cough, nasal discharge and difficulty in breathing. Pneumonia and cough significantly depended on RV. In line with these results, 53% bronchiolitis infections were attributed to RSV [252], and about 30% of pneumonia accounted for RV infections in different studies [266, 271]. In another study, RV were attributable to 40% of pneumonia cases and cough was significantly presented in 86% of the RV infections [241]. Further, in a study cohort examining the influence of a RV infection on wheezing during childhood, a short term increased risk of wheezing after an initial episode of LRTI with was observed [272].

In the present study, a diagnosis of RTI was associated with HAdV and influenza virus. Remarkably, an infection with influenza virus was not associated with fever but correlated well with cough and nasal discharge. The symptoms of influenza virus infection in outpatients are well described [273, 274]. ILI includes fever and cough or sore throat. In a retrospective case-control study among hospitalized ILI patients, cough, coryza, sore throat, and fever were identified to be more common in patients with an influenza virus infection [275]. In a different study, a symptom triad of cough, headache, and pharyngitis was found to be a predictor of influenza virus infection in febrile children [276]. As determined in this and other studies, influenza virus is less detected in patients with ALRI. This is not unusual since influenza viruses usually cause Influenza. For Influenza or ILI the clinical presentation are well described. Therefore it is not surprising that the clinical presentation of influenza virus in ALRI patients may be distinct from the clinical presentation of influenza virus in other patients; however data is scarce. In some investigations, fever and cough was high among ALRI patients but the association to any particular virus has not been assessed [21, 243, 247].

HAdV was associated with RTI and diarrhea in this study. However the high number of HAdV coinfections (56%) with other respiratory viruses may overestimate the role of HAdV in these diseases. Frequently reported clinical signs of HAdV in ALRI included cough, fever and muscular, gastrointestinal symptoms, tonsillitis, bronchitis and pneumonia [58, 277, 278]. These were not significantly associated in the patients of the present study. Interestingly 33.6% of ARI patients from Malaysia were infected with HAdV and had a symptom of diarrhea [279]. Moreover, diarrhea was significantly associated with HMPV in patients with ALRI from this study. So far, diarrhea has rarely been reported among HMPV infections and such association from this study is quite unexpected. In a retrospective analysis of data from a rural setting in Ghana, an association between diarrhea and ALRI was found [280]. However no etiological agents were identified. Other studies as well have indicated diarrhea as a high risk factor for ALRI among young children in low income settings [281, 282]. It may therefore be extrapolated from this study that HMPV could be a possible agent causing diarrhea in patients with ALRI.

HPIV was only significantly associated with fever but not with bronchopneumonia, bronchiolitis or any other RTI in this study. This is remarkable because predominantly HPIV3 which was the most defined species in this study is more likely to cause bronchiolitis and pneumonia in two-thirds of children in the first year of life [47]. In total, 53 and 88 of the

patients presented with bronchiolitis and pneumonia, respectively. Of them, only 16 were infected with HPIV and seven with HPIV. Meaning within the ALRI study cohort there were more likely other pathogens like RSV and RV causing bronchiolitis and pneumonia, and therefore influence the association between these diagnoses and HPIV.

Additionally, comorbidities were found among 10% of studied children. The most commonly observed comorbidities included malaria and skin sepsis. Malaria alone contributed to more than one-third of all comorbidities (20 malaria/53 comorbidities). This is however probable as malaria is endemic in Ghana. Moreover malaria may present with symptoms similar to ALRI [283]. It is therefore plausible that patients with malaria and ALRI symptoms are included in this study: Indeed, 10 of 20 (50%) patients with malaria were positive for one virus.
Further, predisposing conditions for ALRI such as malaria, HIV infection and underlying medical conditions such as cardiovascular disease, chronic obstructive pulmonary disease, asthma, diabetes and cancer or tumor have been reported to play a major role in the disease burden among children especially infants less than one year old [245, 282, 284]. Moreover, non-microbial and socio-economic factors might influence the development of ALRI [282, 285, 286]. These include infant feeding practices, prematurity, exposure to smoke of wood or cigarettes, malnutrition, nutritional status and overcrowding.

In general, it is difficult to distinguish between respiratory viruses in ALRI on clinical grounds only due to the wide range of similar symptoms and co-circulation of these viruses [73, 287]. Specified surveillance systems and virus-diagnosis/symptom associated analyses reduce the amount of possible agents, but in most of the cases differential diagnoses are necessary to identify the pathogen.

4.4 Molecular epidemiology of respiratory viruses inducing ALRI

4.4.1 Influenza viruses

Influenza viruses were identified in 6% of patients during the study. The differentiation data of the virus types showed that influenza A and B viruses were co-circulating in the community during the study. Influenza A(H3N2) virus was detected more frequently and accounted for 63% of infections, A(H1N1)pdm09 virus (6%) and influenza B virus (31%). A review of SARI-associated deaths identified from influenza surveillance during 2009-2012 was reported for

eight sub-Saharan African [13]. In three of these countries involving Kenya, Madagascar and South Africa, influenza virus accounted for 6% of deaths in children aged 0-4 year.

An influenza surveillance study report in Niger from 2009-2013 demonstrated that, 9% of patients aged 1-4 years with SARI tested positive for influenza virus, and influenza A(H1N1)pdm09 viruses were mostly identified [248]. In a similar study from Gabon between 2010-2011, influenza A(H1N1)pdm09 viruses prevailed in 6.8% of infections among children 0-4 years [249]. The predominance of influenza A(H1N1)pdm09 viruses over other influenza virus subtypes was as well reported by a study from Ghana [238]. The investigators documented that particularly among children below 11 years with ILI, the pandemic strain accounted for 86% of all type A cases identified in 2010. The difference observed in the prevalence of the influenza virus subtypes from this study could be explained by the different study periods and populations. However in a study from Togo, an overall predominance of influenza A(H3N2) viruses in 7.4% infections was reported between 2010-2012 [250]. Another study from China also demonstrated that between 2010-2012, 5.9% of hospitalized children with LRTI were positive for influenza virus as follows: 60.65% were influenza A(H3N2) viruses, 12.04% were A(H1N1)pdm09 viruses and 27.31% were influenza B viruses [288].

Influenza A(H3N2) viruses evolving since 2009 have been classified into seven genetic groups [289]. The A(H3N2) viruses circulating in 2013 (from this study) belonged to the genetic clade 3C.3, and were genetically similar to the A/Texas/50/2012 vaccine strain recommended by the WHO for the 2014–15 seasons; however for the majority of them, their antigenic identity did not corresponded with their genetic characterization. Most influenza A(H3N2) viruses circulating during the same time period in other parts of the world revealed similar genetic characteristics [289].

However, there was evidence of co-circulation of four ancestor and/or intermediate A(H3N2) viruses of the genetic group 3C.3 from the present study. In the NA phylogeny, these ancestor viruses clustered differently to the genetic subgroup 3C.3, suggesting reassortment of the indigenous A(H3N2) viruses. While co-circulation of different influenza A(H3N2) viral lineages within the same and/or different influenza season(s) have been shown in other regions to increase the chance for genetic reassortment [290, 291], much is not known for Africa. In an earlier study from New York State (USA), a characteristic multiple co-circulating clades with A(H3N2) viruses was observed [292]. Furthermore, the study revealed multiple reassortment events occurred among the co-circulating clades such that, one clade of H3N2 viruses present

at least since 2000 had provided the HA for all those H3N2 viruses sampled after the 2002–2003 influenza season. A study from Cambodia during 2009-2011 demonstrated that circulating A(H3N2) strains clustered each year to a distinct group and drifted from A/Brisbane/10/2007-like in 2009 to A/Perth/16/2009-like in 2010 and 2011 [291]. In a long-term molecular epidemiology study of A(H3N2) viruses in Mexico from 2003-2012, different A(H3N2) viral lineages were found to co-circulate within the same season and persist between different influenza seasons [290]. Their results pointed out to the circulation of two distinct viral lineages in Mexico during the 2005–2006 seasons: the N-lineage and Brisbane cluster. Further, the Mexican viruses observed within the N-lineage belong to two consecutive seasons (2004–2005 and 2005–2006), and the Brisbane cluster circulated during three influenza seasons (2006-2009).

An influenza A(H3N2) strain from Ghana in 2010 clustered with the 2011-2012 seasons viruses of HA group 3A. Another strain from 2012 clustered with viruses from the season 2013 of the HA subgroup 3C.3. These viruses seemed to have circulated ahead of the rest of the viruses from the same year. Early circulating influenza strains has been described as ‘heralds’ that predetermine dominant strains of a forthcoming season [293]. In a study from Germany, five ‘herald strains’ of A(H3N2) between 2009 and 2012 were identified of the HA groups 3, 4 and 7 [294]. In a global context, different models for the origin of epidemic influenza strains is characterized [295]. For example, it has been suggested that influenza A(H3N2) virus epidemics in the temperate regions do not persist locally between epidemics but were seeded each year from South-East Asia [296]. The ‘sink-source model’ suggests that the genomic evolution of influenza A virus is characterize by a complex interplay between frequent reassortment and periodic selective sweeps from a persistent influenza virus reservoir in the tropics [297]. The epidemic percolation network ‘mathematical model’ also states that new strains of influenza viruses spread around the globe through international air-traffic movement of infected individuals [298]. The frequent identification of ‘reassortants’ and ‘herald’ viruses among influenza A(H3N2) viruses circulating in Ghana suggests that, local virus strains have the potential to evolve into divergent or novel influenza virus lineages, and may contribute to A(H3N2) virus evolution in Ghana.

Influenza A(H1N1)pdm09 viruses since their occurrence in 2009 have evolved and eight genetic groups have been designated based on the HA genes [289]. In recent times, viruses of

genetic group 6 have predominated worldwide with the three genetic subgroups 6A, 6B and 6C [289]. The most recent viruses from Ghana circulating during 2015 fell into the subgroup 6B. The two viruses characterized from this study carry HA genes clustering in genetic subgroup 6C. These two viruses were antigenically similar to the vaccine virus A/California/07/2009. Moreover, similar genetic clustering has been observed for A(H1N1)pdm09 viruses circulating worldwide during these time periods [289, 299].

Two representative viruses from Ghana in 2012 and 2013 clustered with the 2010 pandemic viruses, and this may point toward a local persistence and /or independent lineage evolution. The viruses circulating in 2011 and 2012 fell into two genetic groups 7 and 8. The genetic group 8 has previously been described as unique to West Africa countries including Ghana [300, 301]. These investigations also suggested that substantial viral diversity circulates within Africa and raised questions about the roles of reduced air traffic and the asynchrony of seasonal influenza epidemics amongst West African countries [300].

Two major genetically and antigenically distinct influenza B virus lineages, B/Victoria-lineage and B/Yamagata lineage have been established since 1983 [302]. In recent years, co-circulation and recurring outbreaks of the two lineages have been reported in many regions of the world [301, 303]. All the influenza B viruses from this study belonged to the B/Victoria-lineage. Genetic analysis of the HA gene sequences indicated that they belonged to the genetic clade 1A. The virus strains were closely related to the vaccine virus B/Brisbane/60/2008 that was recommended by the WHO for use in the influenza quadrivalent vaccines [289]. Formerly circulating HA clade 1B viruses from Ghana inherited their NA genes from clade 1A suggesting an intra-clade reassortment between the two genetic subgroups. An inter-clade reassortment between genetic clade 1A/clade 3 was also observed for a reference virus from Ghana. In a recent WHO report, inter-clade reassortment between HA-1B and NA-4, and HA-1A and NA-3 was described for some virus isolates which circulated mainly in China and other Asia countries in 2012-2013 [301].

In summary, multiple lineage co-circulation, persistence and frequent reassortment of influenza viruses underscore the importance of continued epidemiological monitoring and genomic analyses for future influenza surveillance.

4.4.2 Respiratory syncytial viruses

RSV was detected in 23% (127/552) of the children with ALRI. Infections were caused both by RSV group A and B with RSV group B predominating in during the season 2006 and 2013 while RSV group A prevailed during the season 2014. With the exception for the season 2014, RSV group A and B viruses were co-circulating. Similarly, many studies including reports from Kenya [304], Uruguay [305], Malaysia [306], Germany [66] and Japan [130] have shown that RSV group A and B co-circulate with various patterns of group dominance. For example in a retrospective four seasons from 2009-2012 in India, RSV group B predominated and co-circulated with group A in the first two seasons, whereas only group A viruses were found to be in circulation during the third season and predominantly co-circulated with group B viruses in the fourth season [109]. From South Africa RSV group A predominated and co-circulated with group B during 2006-2009 seasons, and alternately in two of four seasons between 2009 and 2012 [101]. Molecular surveillance of RSV in Belgium for 15 consecutive seasons (1996–2011) revealed a shift from a regular 3-yearly cyclic pattern into a yearly alternating periodicity where RSV group B is replaced by RSV group A [120]. The variations in RSV group dominance may suggest a localized nature of RSV circulation in various geographical settings and seasons.

A specific circulation pattern for RSV group A and B could not be observed on the current data for Ghana, at least one following season has to be analyzed. Nonetheless, regular shifts of group dominance have been observed and they have been correlated, in part with variability in the G-protein gene [64].

A total of 46 RSV group A viruses from this study were characterized and belonged to three genotypes. The majority of viruses belonged to the ‘novel’ genotype ON1 (40, 87%). Other genotypes identified were SAA2 (1, 2%, 2006) and NA1 (4, 9%, 2013; 1, 2%, 2014). ON1 predominantly circulated in 2013 and to a much higher extent in 2014, accounting for 76% and 96% of RSV-A infections, respectively. The predominance of ON1 in this study is in agreement with several recent reports worldwide. It was observed that from its first detection in 2010 onwards, the ON1 genotype was rapidly spreading as the dominant RSV-A genotype in 62-94% of the RSV population [106, 112-116]. It seems that ON1 replaces the genotype NA1 in Ghana which is in accordance with reports from different countries describing an intense circulation of genotype NA1 between 2006 and 2012 [101, 103, 110, 117-119]. The predominance of ON1 in the present study further emphasizes the rapid spread of this emerging RSV strain.

Nucleotide and more important amino acid changes directly influence the evolution of viruses. In the present study, the overall divergence within the ON1 genotype was higher than the NA1 in the second hypervariable region of the G protein gene. The ON1 intragenotype divergence was 1.5% at the nucleotide and 2.7% at the amino acid levels. The divergence determined for NA1 was 1% and 0.9%, respectively. Since amino acid divergence was greater than the nucleotide divergence for the ON1 genotype, mutations in the nucleotide resulted in amino acid changes for these genotypes. In addition, corresponding proportion of synonymous and nonsynonymous mutations was observed indicating neutral selective pressure in this gene region of the ON1 viruses. Selective pressure by the immunological response has been described as one of the mechanisms that drive genetic variability of RSV [65, 98]. The higher nucleotide and amino acid variability of the ON1 viruses may have contributed to their rapid dissemination and predominance over the NA1 viruses during the study period. Moreover, the 72-nucleotide duplication might have provided an evolutionary advantage to the ON1 genotype [105].

Detailed analysis of the deduced amino acid sequences of ON1 viruses indicated that all the study sequences differed in many of their amino acid positions from their recent ancestor (Genbank accession number M114486). All the sequences acquired the P274L except for seven of the sequences in which this amino acid was conserved. The 274 amino acid position is a positively selected site that had been previously detected in NA1 variants [102, 120, 306], and in ON1 variants [101, 110, 112, 115, 307]. Additionally, the ON1 viruses of this study demonstrated some unique amino acid substitutions I243S, M262K, N273H, S280H and -298P. These specific substitutions distinguish from the original ON1 reference strain ON67-1210A and other ON1 strains (Fig. 3.10). Notably, amino acid position 262 was detected as a positively selected site among RSV group A viruses from South Africa [101]. Similarly, position 280 has also been reported as a positive selected site for RSV group A viruses elsewhere [143]. However in a recent study from Italy, position 280 was reported as a negatively selected site among RSV group A viruses [115]. The amino acid change -298P is within the duplication region. Probably, the 298 position referred to the same positively selected position (274) of the parent region [143]. The viruses carrying these unique substitutions grouped into different subclusters in the phylogenetic tree. Subclusters with a bootstrap value below 70% indicated the presence of few unique substitutions. Three viruses acquired all five described amino acid mutations and clustered separately with a high bootstrap value of 87%. This distinct cluster suggests that these viruses were evolving more rapidly and

could eventually lead to a new ON variant or genotype. In a comparative study substitutions L274P, L298P, Y304H, and L310P were shared by most ON1 viruses, and defined two major branches of a phylogenetic tree [112].

RSV glycosylation is an important hallmark of antigenicity of the virus. It can mask or facilitate recognition by antibodies of the immune response [308]. Analysis of potential glycosylation site defined amino acid positions 237 and 318 potentially N-glycosylated within the three RSV group A genotypes SAA1, NA1, and ON1. Also positions 251 and 244 in NA1 and SAA2 genotypes are potentially N-glycosylation sites. Interestingly, amino acid T253K substitution led to loss of a potential N-glycosylation site in ON1 strains, and the N273H mutation led to loss of a potential N-glycosylation site for both ON1 and NA1 viruses. Among the ON1 viruses, 37-41 O-glycosylation sites were observed as compared to the 32 residues for NA1 and SAA2 viruses. Previous studies from Canada [105], India [109] and China [110] reported a less number of N- and O-glycosylation sites for genotypes ON1 and NA1, suggesting that glycosylation of the G protein gene is highly variable between RSV strains.

Characterization of 61 RSV group B viruses showed that all viruses belonged to the BA9 genotype. Except for one virus (GHA/RV253/2013) detected in 2013, which belonged to the genotype SAB4. The BA9 genotype predominantly circulated in Ghana in 2006 and 2013, but completely disappeared in 2014. The BA9 genotype was first described in Japan during 2006–2007 [124]. Dominance of the BA9 among the RSV-B genotypes has since been reported worldwide in 66-98% of the RSV population [101, 106, 114-116].

The nucleotide divergence determined for the BA9 genotype was 2.9% and the amino acid divergence was 5.7%. The higher level of amino acid changes compared to nucleotide changes, and additionally the neutral selective pressure for the BA9 viruses may have consequently contributed to their continued circulation in Ghana in 2006 and 2013.

Deduced amino acid sequences analysis for the BA9 viruses demonstrated a number of unique amino acid mutations in comparison to their most recent ancestor (Genbank accession number M17213). Eight specific amino acid substitution (P219R, K233I, L237I, -273D, I281T, L286P, S291L, T302I and P306L) were identified for BA9 viruses from this study. The amino acid position 219 was previously shown to be under positive selection pressure among RSV-B viruses from Italy [115]. Notably, viruses which acquired these specific substitutions fell into

various subclusters. Subclusters with bootstrap values below 70% indicated the presence of few mutations. Subclusters with bootstrap values over 80% indicated the viruses acquired a minimum of three specific mutations. It was interesting to note that the amino acid substitution L237P was reverted to that of the RSV-B reference strain (18537) for a group of viruses. This group of viruses was found to be identical in the phylogenetic tree and formed a separate subcluster. Moreover, position 237 was identified to be under diversifying positive selection in another study [120]. Additionally, positive selection results in frequent reversible amino acid replacements in the G protein gene of RSV and may influence the expression of some important epitopes [309], a phenomenon which probably could be irreversible. The amino acid reversions are likely responsible for the loss of protective immunity that may have been evoked against key epitopes [309]. Ultimately, four subclusters with bootstrap values above 80% were observed in the phylogenetic tree. A number of genetic subgroups for the BA9 genotype has been demonstrated by studies from South Africa [101], India [109] and Italy [115], Germany [106] and Spain [116]. Globally, the G protein gene of the BA9 genotype may be undergoing diversification.

There are some amino acid substitutions resulting in the gain or loss of potential N- or O-glycosylation sites. The amino acid substitutions S296N, P311S and N312T resulted in the gain of two potential N-glycosylation sites for the majority of BA9 viruses and the SAB4 virus. It was interesting to find that the amino acid change D273N resulted in the gain of an additional potential N-glycosylation site for five BA9 viruses. Three other viruses gain an additional potential N-glycosylation site due to the amino acid residue D253N. These two substitutions represent the same position within the parent strain and the duplicated region. Amino acid mutations at positions 296, 311 and 312 were previously reported in other studies to cause a gain of N- glycosylation sites [106, 109, 115].

The number of potential O-glycosylation sites for the majority of the BA9 viruses varied between 40 and 44 predicted sites. For six BA9 viruses, an additional predicted site was observed at amino acid position 317. This was because these six BA9 viruses had a longer protein length of 319 due to the late stop codon at position 320. Generally, a protein length of 312 was dominant for the majority of the BA9 viruses which had a stop codon at amino acid position 313. The SAB4 virus also showed a longer G protein length of 319 as compared to 315 in the SAB4 reference strain Cam2009-1013 (Genbank accession number JN119979) from Cambodia. A range of 35 to 47 O-glycosylation sites were predicted among the BA9 genotype

by other studies [106, 109, 115]. The N- and O-glycosylation of the G protein is suggested to allow viral variants to evolve and escape the immune recognition of their host [308].
The molecular characterization of RSV confirmed the co-circulation of multiple genotypes of both RSV group A and B during the study. The duplication in the G gene of genotype ON1 and BA9 may have given them an evolutionary advantage over other genotypes of RSV-A and B, respectively.

4.4.3 Human metapneumovirus

Compared to other viruses in this study, HMPV was less frequently detected in 3% of patients. Patients were primarily infected with HMPV of the genetic lineage B1 and B2, and the sub-genetic lineage A2a. Seasons 2006 and 2013 were presented by only one genetic lineage namely B1 and B2, respectively. In the season 2014 co-circulation of B2 (71%) and A2a (29%) was observed while the genetic lineage B2 was predominating. At a similar time between 2007/2008, patients with LRTI from Egypt were infected with the genetic lineage B2 (85%) and B1 (15%) [149]. In South Africa lineage B2 (73%) was dominant during 2000 and co-circulated with lineages A1 and A2 during 2001 [310]. In Croatia between 2005/2006, 50% of HMPV detected among patients during a 1-year study belonged to lineage B2 and co-circulated with all HMPV lineages B1, A1, A2a and A2b [311]. Differently for the period of 2002-2007 in Brazil all four HMPV genetic lineages circulated, A2a in 2007, B2 in 2006, B1 and B2 in 2004, and A1 and A2a in 2003; no co-circulation was found between the genetic lineages of A and B in the same year period [150]. However during 2005/2006 in India, co-circulation of A2b and B1 was found with A2b predominating, whereas between 2006/2007 no lineage A viruses were detected but instead co-circulation of B1 and B2 viruses was seen with B1 viruses predominating [148]. During 2009-2011 in Kuwait, A2b predominantly circulated with B2 [312]. In long-term European studies from France (2002-2009) [146] and Germany (2000-2010) [145], it was observed that the prevalence of the HMPV subgroups and genetic lineages is fluctuating by year, giving rise to frequently observed switching of the predominantly circulating group.

The amino acid sequences show stronger similarity than the nucleotide sequences. Meaning mutations of nucleotides do not regularly result in amino acid changes of the virus. Additionally for both B2 and A2a viruses, nonsynonymous mutations were less frequently than synonymous mutations observed suggesting a negative selection pressure on the HMPV

viruses. Seemingly, the purifying negative selective pressure may have contributed to the HMPV genomic stability to resist appearance of deleterious mutations and genetic diversity [309]. Besides, the high stability of the amino acid sequence among A2a viruses may signify higher susceptibility of A2a to host immune pressure [143], resulting in subsequently low circulation and detection of this sub-lineage during the study.

Analysis of the deduced amino acid sequences revealed subgroup related mutations. Substitutions N233Y, V286I and Q312K were unique for HMPV subgroup B viruses, and D296K and E348K were for subgroup A viruses. Despite the high amino acid concentrations among HMPV mutations, T223N and D280N have been exclusively detected in HMPV genetic lineage B2 sequences from this study but not found in other B2 sequences from elsewhere. The additional substitutions confirmed the separation of these HMPV B2 viruses into a distinctive subcluster in the phylogenetic tree (Fig. 3.12). However, these specific amino acid changes resulted in no gain of a potential N-glycosylation. Their biological importance among the B2 lineage is so far not clear.

4.4.4 Human Adenoviruses

HAdV cause a variety of diseases worldwide. They are grouped into six species [84] causing species specific infections. Species B, C or E usually cause respiratory diseases [159]. Interestingly, children with ALRI from the present study were infected by HAdV from each of the six species A-F. HAdV species B (21/64, 33%) and C (28/64, 44%) were most commonly detected. Other HAdV species were detected at equal amount (A and E each 5%; D and F each 6%). All the HAdV species, either as single or coinfections were involved in respiratory tract infection, and except for HAdV species E all the other species frequently caused diarrhea symptoms in the patients. In line with reports from tropical and subtropical regions, for example Brazil [299], Thailand [160], Peru [55], Malaysia [165, 313] and Egypt [161], HAdV species C was the predominant HAdV circulating in these countries. However investigations from temperate regions like Taiwan [58], Argentina [163], Korea [166] and China [162] have generally reported HAdV species B to be the predominant species identified. It is reported that HAdV species A-D, and F can cause an array of clinical diseases including gastroenteritis [159]. Most of these occur in children younger than five years old and are generally self-limiting illnesses.

For the period of 2013, all the six different HAdV species [39] co-circulated as compared to one species C in 2006 and four species B, C, D and F in 2014. Different distribution patterns have been observed previously for HAdV species. During an 8-year study period (2003-2010) in Egypt [161] and a 5-year study period (2006-2010) in Peru [55], HAdV species B, C and E were identified among patients with ILI and SARI. In both studies, co-circulation of HAdV species B and C was observed each year, while HAdV species E circulated sporadically in year 2009. In a 4-year study period (2009-2012) from Thailand [160] four HAdV species B, C, D and F were identified among the study population. However from Malaysia [165], only HAdV species B and C circulated during a 9-year study from 2003-2011. Diverse geographical locations may play an important role in HAdV species prevalence, predominance, distribution, and circulation pattern.

The sequence analysis of the hexon and fiber protein genes revealed that 13 distinct HAdV types circulated during the study. Within the predominating species B and C each, 2 (B3, B7) and 5 (C1, C2, C5, C6, and C57) types were identified. Of all types, B3 (n=8) and C1 (n=11) were most prevalent. Generally in most reports, HAdV types C1, C2, B3, C5 and B7 were frequently detected from children with ALRI [58, 165, 314]. HAdV-B3 and HAdV-C1 respectively accounted for 31% and 32% of infections over the 4-year study from Thailand, and in 2009 of the study HAdV-C1 accounted for more than 50% of infections [160]. Likewise in the study from Egypt [161], HAdV-B3 and HAdV-C1 accounted for equal proportions and represented the majority of infections. In the study from Peru, HAdV-C1, 2, 5 and 6, and HAdV-B3 and 7 were commonly identified [55].

Recombination is a recognized feature of HAdV which may lead to the emergence of new types and subtypes [154]. Notably from this study, three HAdV-B7 viruses analyzed by the hexon gene had their fiber gene sequences aligned closely with the HAdV-B3 reference strain instead of the HAdV-B7 reference sequences in the phylogenetic tree, indicating derivation of their fiber gene from the HAdV-B3 viruses. However confirmation of this event requires the sequencing of the complete genome. This finding supports other observations that recombination events may normally occur between strains of the same species, and interspecies recombinants are uncommon [157, 162, 315]. It is suggested that viral genetic diversity caused by recombination was a main source of emerging outbreaks [153]. For example in 2014 in Taiwan, outbreak of adenovirus was predominantly constituted by HAdV-B3 (72%) and HAdV-B7 (15%) [58]. The hexon protein gene sequences were highly conserved for HAdV-B7 circulation in Taiwan, but the fiber gene in HAdV-B7 shifted from 7b to 7d. In a different report

from Argentina, a highly virulent and predominant HAdV-B7h was clearly indicated as an emerging virus resulting from the recombination of HAdV-B3 fiber gene [316]. Recently, an outbreak involving a new recombinant strain (HAdV-B7/HAdV-B3) containing HAdV type 7 hexon and type 3 fiber genes was associated with fatal infections among infants from Portugal [158]. During the last decade, several other outbreaks of severe infections have been frequently reported for HAdV-B7 [164, 317, 318] and HAdV-B3 [57, 62]. For this study, only three recombinant HAdV-B7/HAdV-B3 viruses were identified in 2013 and 2014. If these recombinant viruses were relicts from the past or precursor of an upcoming HAdV outbreak in Ghana cannot be concluded, since molecular data for these pathogen are lacking.

This study highlights the co-circulation of multiple HAdV species and types in patients with ALRI. The presence of newly emerging recombinant types or variants of HAdV underscore the need for constant and close surveillance.

4.4.5 Human Rhinoviruses

In this study, RV were the second most pathogen inducing ALRI in children less than five years old. The phylogenetic analysis revealed that patients were infected with all known RV species A-C. The majority of RV belonged to RV species A and C with 46% and 48%, respectively. These two species co-circulated in all the three seasons, i.e. 2006, 2013 and 2014. RV species B viruses were detected in 6% of the samples and circulated only during 2013. A comparable distribution pattern was described by investigators from Kenya [177]. Among 298 samples from inpatient children with ALRI, 47% was classified as RV species A, 4.4% as RV species B, and 48% as RV species C. Additionally in South Africa [181] and Tanzania [319], RV species A was the most prevalent in 48% and 52% of the samples, respectively. However, all three reports demonstrated a year-round co-circulation of RV species A and C, and a sporadic detection of RV species B.

Remarkably, the phylogenetic analysis revealed circulation of 40 different RV types among ALRI patients: 16 RV-A, 4 RV-B and 20 RV-C. The individual RV types showed considerable variability in the detection frequency. The most frequent types were RV-C2 (n = 9) and RV-A12 (n = 6). Interestingly, RV-A12 only induces (broncho-) pneumonia in children from this study. RV-C2 was investigated in patients with different diseases including bronchopneumonia (n=4), respiratory tract infection (n=2), respiratory distress (n=2), and bronchiolitis (n=1). Further, the majority of RV types (n = 30) were identified in 2013 and circulated only in that

year except for three types: RV-A12 detected in 2006, 2013 and 2014; RV-C23 in 2006 and 2013, and RV-C39 in 2013 and 2014. Similarly to this study, a significant genetic diversity of RV was also observed in Tanzania [319]. There, a high number of 50 different RV types were detected in 2008 with RV-A12 and RV-C2 prevailing. Moreover, a comparison of RV dataset for various countries suggested that RV-A1, A12, A49, A78, A101, RV-B69, RV-C2, C6, C16, C43, and Cpat18 show higher prevalence than other RV types [171]. The large diversity among RV species has been attributed to recombination events [74]. For example, recombination between RV-A53 and RV-A80 sequences resulted in the emergence of a third RV-A46 [167].

Further, seven divergent RV variants of RV species A and C were identified in this study. Relating to their pairwise p-distance above 10.5% within the VP4/VP2 sequences, the divergent RV variants were named as provisionally assigned types (PATs), namely as RV-Apat_a, RV-Apat_b, RV-Cpat_a, and RV-Cpat_b. Nonetheless for these viruses, the VP1 genes have to be sequenced to confirmed them as novel types [171]. The putative new PAT types probably confirm the high genetic variability among RV species A and C. Importantly, amongst recent studies from Tanzania [319], Cambodia [271], China [173] and Australia [320], divergent unassigned RV variants or PATs have been reported. However in light of the many newly assigned PATs from different reports, a difficulty was encountered in tracking and systematically assigning numbers to the PATs in this study. It is recommended that if possible, the Picornaviridae Study Group should as well oversee the assignment of new PATs and periodically make official updates available.

The relative prevalence and distribution of the RV types identified in this present study is characteristic for the different but interactive circulating pattern worldwide [171]. There is the need for future studies which would identify and expand upon the genomic characteristics of RV in Ghana.

5 Concluding remarks

The present study described the contribution of respiratory viruses to the burden of ALRI within the pediatric population in Ghana, which accounted for 73% of respiratory infections during the 3-year period investigated. RSV (23%), RV (19%), HBoV (14%), HPIV (12%), and HAdV (12%) were found to considerably contribute to ALRI pathology.

The prevalence of the viral pathogens varied across the categories of age groups studied. For example, infants were mainly infected with RSV (33%), toddlers with HBoV (12%), and preschool children with influenza viruses (22%). Moreover, respiratory viruses each circulated in the dry and /or the rainy season, which caused enhanced ALRI activity in February, July and October each year. These observations are intriguing and demonstrate that the implementation of a children based ARI/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.

For most respiratory virus pathogens circulating in Ghana, molecular characterization data is missing. Besides influenza virus and RSV, this study provides additional molecular and phylogenetic information on HMPV, HAdV, and RV. RSV and HMPV demonstrated a stable and dominant circulation of BA9 genotype and B2 genetic lineage, respectively within the study period. HAdV and influenza virus showed yearly displacement of dominant subtypes and types. RV displayed a broad genetic diversity with many strains circulating in a single year only. The phylogenetic analyses were in part based on a small number of samples, suggesting that for these viruses, e.g. HMPV or influenza virus, the observed variability could be underestimated. Nonetheless, co-circulation of several virus strains was observed. To investigate the persistence, displacement or new emerging virus strains, consecutive or pathogen-related long-term molecular studies are needed. 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 so far unanswered questions of viral evolution, and they may be of further interest for vaccine development, especially needed in developing countries.

Although virus-associated clinical diagnoses were observed in this study, there were clinical manifestations to be present in infections with different viruses, thus making it difficult to distinguish between causative agents on clinical grounds alone. The ability to ultimately

identify the viral etiology of ALRI and differentiate between these infections is fundamental to effective treatment if available. Differential diagnosis could therefore support patient management and prevent and/or control infections within a hospital setting. In addition, routine testing may serve for rapid detection of epidemics of respiratory virus infections, responses to outbreaks and resource allocation.

The present study has limitations. These include potential sampling biases that may affect positivity rates. In this study, the total number of samples was highest in 2013 compared to 2006 and 2014. In 2014, still all incoming patients were enrolled, but the number of patients was several times lower than in 2013. A reason could be that in 2014 other hospitals were upgraded to a tertiary care facility in the Greater Accra Region, and therefore the catchment area has changed. Moreover, the investigation period was discontinuous. This complicates the interpretation of the molecular epidemiology of virus strains and the time-dependent circulation of the respiratory viruses. Also comparison to other studies was limited by the fact that different populations or periods of the year were examined. Nonetheless, this study still enables the description of both circulating virus strains and in tendency of circulation patterns, and more important provides first molecular and phylogenetic information on respiratory viruses from Ghana. However, further hospitals should be included in future studies to ensure a reliable sample number. Further, the investigation period should be extended to at least four continuous seasons, because there are viruses, e.g. HPIV which generally have a biannually circulation pattern. Malaria was documented as a leading comorbidity among ALRI patients, however laboratory confirmation of malaria diagnoses was not part of this study. Moreover, comorbidities such as HIV infection, malnutrition and parasitic infection status for the patients enrolled in this study were unknown. Patients particularly with HIV infection may have prolonged shedding of viral pathogens and a different spectrum of viral ALRI. These could possibly influence the outcomes of this study if such cases were analyzed. The need for further investigation with asymptomatic subjects is equally necessary to fully explain the unique role of some respiratory viruses including RV, HAdV, HBoV and HCoV associated with ALRI in Ghana. Prospective studies should in addition focus on other factors such as viral load, pathology and host interactions of these viruses to describe their outcome in the pediatric ALRI burden.

6 Summary

Acute lower respiratory tract infections (ALRI) cause annually more than one million deaths in children under the age of five years worldwide. This accounts for 18% of all childhood mortality, of which 99% occurs in developing countries. In Ghana, ALRI, particularly pneumonia, accounted for an 11.3% increase in hospital admissions with a case fatality rate of 38% in 2011. However, collection and analysis of surveillance data is rarely performed and limited to a few pathogens.

Therefore from February 2006 to November 2006, and January 2013 to December 2014, children with ALRI between 0 and 5 years of age were prospectively enrolled from two hospitals in Accra, Ghana. Children below the age of one year were mostly affected by ALRI. Nasopharyngeal aspirates or nasal swabs were collected from all patients and investigated for 16 common respiratory pathogens by specific real-time PCR assays. Seventy-three percent (404/552) of the specimens were positive for at least one respiratory virus. Beside RSV (23%), RV (19%), HBoV (14%), HPIV (12%), and HAdV (12%) were found to considerably contribute to ALRI. Respiratory viruses each circulated in the dry and/or the rainy season causing enhanced ALRI activity in February, July, and October of each year. Further, infants were mainly infected with RSV (33%), toddlers with HBoV (21%), and preschool children with influenza viruses (22%). The clinical diagnosis of ALRI patients included bronchopneumonia and bronchiolitis, each being highly associated with RSV. Pneumonia was significantly associated with RV, and respiratory tract infection with HAdV and influenza viruses.

To investigate the circulating virus strains phylogenetic analyses were performed. Of RSV group A and B viruses mainly genotypes ON1 and BA9, respectively, caused ALRI. Further, of HMPV genetic lineages A2a, B1, and B2 as well as of influenza virus A(H3N2), A(H1N1), and B/Victoria-lineage clades 3C.3, 6C, and 1A viruses were identified. HAdV species B and C were most commonly detected among other species in these patients. As expected, a high number of RV types including four new provisionally assigned types were identified. RV mainly belonged to species A and C.

This study investigated the role of 16 respiratory pathogens on the viral etiology of ALRI in Ghana. For the first time, comprehensive molecular and epidemiological data were provided including rarely investigated pathogens like HMPV, RV, HBoV, and HCoV. The detection of multiple viruses highlights the need for prospective surveillance and routine diagnostic in order to take protective measures or to improve patient care.

7 Zusammenfassung

Jährlich sterben weltweit mehr als eine Million Kinder bis zu einem Alter von fünf Jahren an akuten Erkrankungen der unteren Atemwege (ALRI). Dies entspricht einer Kindersterblichkeit von 18%; wobei 99% dieser Fälle in Entwicklungsländern auftreten. In Ghana führten 2011 ALRI, insbesondere Pneumonien, zu einem Anstieg der Krankenhauseinweisungen auf 11,3% und zu einer Letalität von 38%. Erregerspezifische Surveillance-Daten werden jedoch kaum erhoben und sind zudem auf wenige Pathogene begrenzt.

Daher wurden von Februar 2006 bis November 2006 und Januar 2013 bis Dezember 2014 prospektiv Kinder mit ALRI im Alter von 0 und 5 Jahren aus zwei Krankenhäusern in Accra, Ghana in diese Studie aufgenommen. Der Großteil der an ALRI erkrankten Patienten waren Kleinkinder jünger als ein Jahr. Von allen Patienten wurden entweder Nasen-Rachen-Aspirate oder Nasenabstriche mit spezifischen real-time PCR-Assays auf 16 respiratorischer Erreger untersucht. Dreiundsiebzig Prozent (404/552) der Proben waren für mindestens ein Atemwegsvirus positiv. Neben RSV (23%) trugen RV (19%), HBoV (14%), HPIV (12%) und HAdV (12%) erheblich zu ALRI bei. Die respiratorischen Viren zirkulierten in der Trocken- und/oder Regenzeit und führten jedes Jahr im Februar, Juli und Oktober zu einer Verstärkung der ALRI Aktivität. Darüber hinaus wurden hauptsächlich Säuglinge mit RSV infiziert (33%), Kleinkinder mit HBoV (21%), und Vorschulkinder mit Influenzaviren (22%). Die klinische Diagnose von ALRI-Patienten beinhaltete unter anderem Bronchopneumonie und Bronchiolitis, welche jeweils signifikant mit RSV assoziiert waren. Pneumonie war signifikant mit RV und Erkrankungen der Atemwege mit HAdV und Influenza-Viren assoziiert.

Um Informationen über die zirkulierenden Viren zu erhalten, wurden phylogenetische Analysen durchgeführt. ALRI wurde von RSV Gruppe A und B Viren hauptsächtlich durch die Genotypen ON1 und BA9 verursacht. Weiterhin wurden von HMPV die genetischen [237] Linien A2a, B1 und B2 sowie von Influenzavirus A(H3N2), A(H1N1) und B/Victoria-Linie die Clades 3C.3, 6C und 1A identifiziert. HAdV Spezies B und C wurden am häufigsten neben anderen HAdV Spezies in diesen Patienten nachgewiesen. Wie erwartet wurde eine hohe Anzahl von RV-Typen, darunter vier neue vorläufig bezeichnete RV-Typen, identifiziert. Diese RV-Typen gehörten vor allem zu den Spezies A und C.

In dieser Studie wurde die Rolle von 16 Atemwegserregern auf die virale Ätiologie von ALRI in Ghana untersucht. Erstmalig wurden umfassende molekulare und epidemiologische Daten einschließlich selten untersuchter Erreger wie HMPV, RV, HBoV und HCoV erhoben. Die Vielzahl der nachgewiesenen respiratorischen Viren unterstreicht die Notwendigkeit für eine prospektive Surveillance und Routinediagnostik, um geeignete Präventionsmaßnahmen zu ergreifen oder die Patientenversorgung in Ghana zu verbessern.

8 References

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Declaration of authorship

I certify that the work presented here is, to the best of my knowledge and belief, original and the result of my own investigations except as acknowledged, and has not been submitted either in part or whole for a degree at this or any other University.

Berlin, June 2016

Signature: _____

Evangeline Obodai

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To God be the Glory!

Curriculum vitae

My curriculum vitae will not be published in the electronic version of my work for privacy reasons.

Poster

Evangeline Obodai, Janine Reiche, Barbara Biere, Thorsten Wolff, Brunhilde Schweiger. Viral pathogens associated with acute lower respiratory tract infections in children from Ghana. 25th Annual Meeting of the Society for Virology 2015, 18-21 March 2015, Bochum, Germany.

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Appendix 1

RV species A sequences compressed in the phylogenetic tree

RV species A	
RV Type	**GenBank accession number**
RV-A1	D00239
	FJ445111
	JN837694
	JN815255
RV-A9	FJ445114
	FJ445115
	FJ445177
RV-A10	DQ473498
	FJ445178
	JN541269
	JN815247
	JN798575
	JN798582
RV-A103	JF965515
	JQ994499
	JQ747749
RV-A12	JF781511
	HQ123441
RV-A13	FJ445116
	FJ445117
RV-A15	DQ473493
	JN541268
RV-A16	JN614992
	JN798574
	JN815253
	JN798564
	JN990704
	JX074057
RV-A18	FJ445118
	JF781496
	JF781508
RV-A19	JQ747746
	JQ747750
	FJ445119
RV-A20	FJ445120
	JN541270
	JN614993
	JN798571
	JQ994494
RV-A21	FJ445121
	JN837693
	JQ747747
RV-A23	DQ473497
	JN621244
	JN815254
	JN837696
RV-A30	DQ473512
	FJ445179
RV-A33	FJ445128
	JN815250
	JN990707
RV-A34	DQ473501
	FJ445189
	JF781510
	JF781512
	JN562720
RV-A36	DQ473505
	JF781497
	JN614994
	JN621243
	JN798583
	JN837697
	JX074050
RV-A38	DQ473495
	FJ445180
	JQ994496
RV-A40	FJ445129
	JN798579
	JQ245967
	JX074051
RV-A43	FJ445131
	JN815237
RV-A47	FJ445133
	GQ223229
	JN837692
RV-A51	FJ445136
	JN562725
RV-A53	DQ473507
	JN798587
RV-A54	FJ445138
	FJ445139
RV-A55	DQ473511
	JQ837718
RV-A57	FJ445141
	JN614995
RV-A59	DQ473500
	JN541266
RV-A60	FJ445143
	JN798590
RV-A65	FJ445147
	JF781504
	JQ245966
RV-A67	FJ445149
	JN621245
RV-A68	FJ445150
	JN798578
RV-A71	FJ445152
	JQ245971
	JX025555
RV-A75	DQ473510
	JF781503
	JN837690
RV-A76	DQ473502
	FJ445182
	JX074049
	JX074055
RV-A80	FJ445156
	JN798576
	JN798586
	JN990705
RV-A81	FJ445157
	FJ445158
	FJ445159
	HQ123442
RV-A82	DQ473509
	FJ445160
	JN798556
	JQ837722
RV-A89	FJ445165
	FJ445166
	JQ837716
	JQ837719
	FJ445184
	M16248

RV species B and C sequences compressed in the phylogenetic trees

RV species B	
RV Type	**GenBank accession number**
RV-B4	DQ473490
	JN798573
RV-B103	JN614996
	JQ994497
	JN798572
	JQ245972
	JQ837717
RV-B14	K02121
	L05355
	X01087
RV-B35	DQ473487
	FJ445187
	JF781501
	JX074052
RV-B42	FJ445130
	JF781498
	JF781507
	JN562724
RV-B48	DQ473488
	JN990698
RV-B52	EF173424
	FJ445188
	FJ445137
	JF781506
RV-B6	DQ473486
	JN562723
	JX193795
	JQ747745
	JQ747748
RV-B69	FJ445151
	JN562721
	JQ245970
	HQ123445
RV-B70	DQ473489
	JQ245974
RV-B72	FJ445153
	GU968948
	JQ245969
	JN614997
RV-B83	FJ445161
	JN990701
RV-B84	FJ445162
	JF781499
	JN614991
	JQ837723
	JN541271

RV species C	
RV Type	**GenBank accession number**
RV-C1	EF077279
	HQ123443
RV-C2	EF077280
	JX025557
	JN815248
	JN990703
	JN837695
	JQ245968
RV-C03	EF186077
	JN798567
	JN990700
RV-C06	EF582387
	JF317016
	JN990702
RV-C08	GQ223227
	JQ245964
RV-C17	JN815240
	JN815244
RV-C25	HQ123440
	JF317013
	JN837685
RV-C32	JN798581
	JQ994498
RV-C40	JF781505
	JN815251
RV-C44	HE993849
	HE993850
	HE993851
RV-C7	DQ875932
	JN837689
	JN798559
	JX025556
	JN798570

Appendix 2

UNIVERSITY OF GHANA MEDICAL SCHOOL
COLLEGE OF HEALTH SCIENCES
ACADEMIC AFFAIRS OFFICE

Phone: +233-0302-666987-8
Fax: +233-0302-663062
E-mail: academic.ugms@chs.edu.gh
My Ref. No: MS-AA/C.2/Vol.16[A]

Your Ref. No.

P O Box 4236
Accra
Ghana

6th August, 2013

Mrs. Evangeline Obodai
Virology Department
NMIMR, UG

ETHICAL CLEARANCE

Protocol Identification Number: MS-Et/M.7 – P 4.10/2012-2013

The Ethical and Protocol Review Committee of the University of Ghana Medical School on 27th June, 2013 unanimously approved your research proposal.

TITLE OF PROTOCOL:"**Molecular Epidemiology of Respiratory Viruses among Children Under 5 Years with Acute Lower Respiratory Tract Infections in Ghana**"

PRINCIPAL INVESTIGATOR: Mrs. Evangeline Obodai

This approval requires that you submit six-monthly review reports of the protocol to the Committee and a final full review to the Ethical and Protocol Review Committee at the completion of the study. The Committee may observe, or cause to be observed, procedures and records of the study during and after implementation.

Please note that any significant modification of this project must be submitted to the Committee for review and approval before its implementation.

You are required to report all serious adverse events related to this study to the Ethical and Protocol Review Committee within seven (7) days verbally and fourteen (14) days in writing.

As part of the review process, it is the Committee's duty to review the ethical aspects of any manuscript that may be produced from this study. You will therefore be required to furnish the Committee with any manuscript for publication.

This ethical clearance is valid till July 2016.

Please always quote the protocol identification number in all future correspondence in relation to this protocol.

Signed:.....
PROFESSOR JENNIFER WELBECK
(CHAIRPERSON, ETHICAL AND PROTOCOL REVIEW COMMITTEE)

cc: Dean
Head of Department
Research Office

Appendix 3

ALRI SURVEY QUESTIONAIRE FORM

FORM NUMBER:……..

ELIGIBILITY: Children aged 0-5years presenting with ALRI, in addition to symptoms of cough or nasal discharge or fever

1. Date of Visit:…… 2. Date of sample collection……

3. Age:……. 4. Sex: M / F

5. Name: ……..……..

6. Hospital Folder No.: ……

7. Address (Place of residence): ……

8. Description of child's condition (Present/Yes = Y, Absent/No = N)

a) Fever: Y / N b) Nasal discharge: Y / N c) Fast breathing: Y / N

d) Cough: Y / N e) Difficulty-in-breathing: Y / N f) Difficulty-in-feeding: Y/N

g) Others (vomiting/diarrhea/abdominal pain etc.) please specify ……..

h) If yes, duration of symptoms: ……..

09. Has the child any medication for treatment of illness: Y / N, please specify: …..…

10. Has your child suffered from any previous disease: Y / N, please state……

11. Feeding history up to six months: a) Exclusive breast feeding b) others, specify

12. Does child sleep alone: Y / N 13. If no, how many in a room: ……

14. N0. Of windows in the room: … 15. Are there any siblings with ALRI: Y / N

16. Is there any form of environmental/passive tobacco smoking: Y / N

17. Educational status of child: a) none b) crèche c) nursery d) primary

18. Educational status of parent: a) none b) primary c) JHS / SHS d) Tertiary

19. Occupation of parent/guardian: ……..

20. Medical Exam: Temp (^{o}C) ……. b) Respiratory rate ……. c) others, state…....

21. Clinical diagnosis: a) Bronchiolitis b) Bronchopneumonia c) RTI
d) Pneumonia e) Respiratory distress f) others…