HUMAN METAPNEUMOVIRUS AND RESPIRATORY SYNCYTIAL VIRUSES
IN GHANAIAN CHILDREN BELOW FIVE YEARS WITH ACUTE LOWER
RESPIRATORY TRACT INFECTIONS IN ACCRA, GHANA

BY

ANNA ABA HAYFORD

(10264096)

A THESIS SUBMITTED TO THE UNIVERSITY OF GHANA, LEGON IN
PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE AWARD OF
MASTER OF PHILOSOPHY DEGREE IN MEDICAL MICROBIOLOGY

JULY 2017
DECLARATION

I hereby declare that this research is a result of my own research work carried out at the Virology Department of the Noguchi Memorial Institute for Medical Research, under the supervision of Professor Theophilus Koku Adiku of the Department of Medical Microbiology, School of Biomedical and Allied Health Sciences (SBAHS) Korle Bu and Dr. John Kofi Odoom of the Noguchi Memorial Institute for Medical Research, Legon. I further declare that, any references used in this thesis that is not mine has been duly acknowledged and that this work has not been submitted to any other institution for the purposes of obtaining a degree.

......................................................... Date.........................
ANNA ABA HAYFORD
(STUDENT)

......................................................... Date.........................
PROF. THEOPHILUS KOKU ADIKU
(SUPERVISOR)

......................................................... Date.........................
DR. JOHN KOFI ODOOM
(SUPERVISOR)
DEDICATION

Dedicated to my beloved family.
ACKNOWLEDGEMENT

A research work of this magnitude would not have been possible without the help of many individuals. I wish to express my profound gratitude to my supervisor Professor Theophilus Adiku who encouraged me to write on this topic. The Professor, despite great pressure of work was of great help to me by taking time off his tight schedule to glance through the work at various stages and offered advice, useful criticism and suggestion and encouragement.

To my external supervisor, Dr. Kofi Odoom, I owe a debt of immeasurable gratitude. He was very helpful with bioinformatics and phylogenetic analysis in this work and for agreeing to be my external supervisor.

Sincerely, I cannot neglect mentioning Dr. (Mrs.) Evangeline Obodai for her immense contribution during the entire work. She assisted in availing to me the relevant laboratory reagents and was exceptionally helpful in answering my many random scientific questions. Dr. Margaret Neizer (Clinician) and Gifty Okine, both of the Princess Marie-Louise Hospital were equally very helpful with sample collection and also deserve mentioning.

To Professor William Kwabena Ampofo, Head of Virology Department and Director of the Noguchi Memorial Institute for Medical Research (NMIMR), Legon, who granted me access to use the facility of the institute, I am most grateful.

Without the cooperation and technical support of the following persons, this work could not have been accomplished; Dr. and Dr. (Mrs) Bonney, Prince Kofi Parbie, Naa Dedei
Aryequaye and Christopher Abana all of the NMIMR who individually and collectively engaged in the sequencing of my samples. In addition, Dr. Gloria Adjapong of the Center for Plant Medicine Research – Mampong (CPMR), also took it upon herself to proof read the whole work and offered me very useful suggestions.

I thank Elijah Paa Edu-Quansah, Yaw Larbi, Prince Asare (NMIMR) and Dr. Calys-Tagoe (Community Health, UG) for helping out with my data analysis.

Finally, my appreciation goes to my husband Kwame, my children, Elorm, David and Elinam, who had to bear my long absence from home. My Mum, Charlotte for her unflinching support throughout my academic life. My prayerful in-laws, Rev and Mrs. Kafintu- Kwashie; for their prayers and supplications which preserved me throughout this academic exercise.

My siblings Getty, Paapa and Ma-Adwoa; My friends Sethina, Nancy, Justice, Nana Aba, Vivian, Leonora and Diana Ahu Prah (NMIMR, Bacteriology Department) for their continuous moral support not forgetting the head and colleagues of the Department of Medical Microbiology especially Dr. Japhet Opithan, Mr Boamah, Makafui and my two wonderful Administrative Secretaries: Ms Evelyn Omane and Ms Emelia Aryettey.
# TABLE OF CONTENTS

DECLARATION .......................................................................................................................... i
DEDICATION .............................................................................................................................. ii
ACKNOWLEDGEMENT ........................................................................................................... iii
TABLE OF CONTENTS .............................................................................................................. v
LIST OF FIGURES ...................................................................................................................... viii
LIST OF TABLES ........................................................................................................................ ix
LIST OF ABBREVIATIONS AND ACRONYMNS .................................................................... x
ABSTRACT ............................................................................................................................... xi

## CHAPTER ONE ..................................................................................................................... 1

1.0 INTRODUCTION ............................................................................................................... 1
   1.1 BACKGROUND ............................................................................................................... 1
   1.2 PROBLEM STATEMENT ............................................................................................... 4
   1.3 JUSTIFICATION ........................................................................................................... 5
   1.4 AIM ............................................................................................................................... 6
      1.4.1 SPECIFIC OBJECTIVES .......................................................................................... 6

## CHAPTER TWO ..................................................................................................................... 7

2.0 LITERATURE REVIEW ..................................................................................................... 7
   2.1 HISTORY AND STRUCTURE OF RESPIRATORY SYNCYTIAL VIRUS ......................... 7
   2.2 MOLECULAR EPIDEMIOLOGY OF RSV .................................................................... 9
   2.3 RSV TRANSMISSION .................................................................................................. 11
   2.4 PATHOGENESIS OF RESPIRATORY SYNCYTIAL VIRUS ........................................ 11
   2.5 CLINICAL PRESENTATIONS ....................................................................................... 12
   2.6 BURDEN OF DISEASE ASSOCIATED WITH RSV ................................................... 14
   2.7 LABORATORY DIAGNOSIS ......................................................................................... 15
   2.8 RISK FACTORS ASSOCIATED WITH RSV INFECTIONS ...................................... 16
   2.9 SEASONALITY ............................................................................................................. 16
   2.10 TREATMENT AND MANAGEMENT OF RSV INFECTION ...................................... 17
   2.11 HISTORY AND STRUCTURE OF HUMAN METAPNEUMOVIRUS (HMPV) ............... 18
2.12 MOLECULAR EPIDEMIOLOGY OF HMPV ....................................................... 20
2.13 TRANSMISSION .......................................................................................... 21
2.14 PATHOGENESIS OF HMPV ........................................................................ 22
2.15 CLINICAL PRESENTATIONS OF HMPV ..................................................... 22
2.16 SEASONALITY .............................................................................................. 23
2.17 LABORATORY DIAGNOSIS OF HMPV ....................................................... 23
2.18 DISEASE BURDEN ASSOCIATED WITH HMPV ....................................... 24
2.19 TREATMENT AND MANAGEMENT OF HMPV ........................................... 24
CHAPTER THREE ............................................................................................. 26
3.0 METHODOLOGY ........................................................................................... 26
3.1 STUDY DESIGN .............................................................................................. 26
3.2 STUDY SITE ................................................................................................ 26
3.3 RECRUITMENT OF SUBJECTS ..................................................................... 27
3.4 INCLUSION CRITERIA .................................................................................... 27
3.5 EXCLUSION CRITERIA ................................................................................... 27
3.6 CASE DEFINITION OF ACUTE LOWER RESPIRATORY INFECTION
(ALRTI) ............................................................................................................... 27
3.7 SAMPLING SIZE ............................................................................................ 28
3.8 SAMPLING METHOD ..................................................................................... 28
3.9 TOOLS AND TECHNIQUES FOR SAMPLE AND DATA COLLECTION .......... 29
   3.9.1 Questionnaire .......................................................................................... 29
   3.9.2 Sample collection/Transportation /Storage ........................................... 29
3.10 LABORATORY INVESTIGATION/ PROCEDURE ........................................... 30
   3.10.1 Sample preparation (RNA extraction) ................................................ 30
3.11 Reverse TRANSCRIPTION (RT) of viral RNA ............................................. 31
3.12 CONVENTIONAL PCR ................................................................................ 31
3.13 GEL ELECTROPHORESIS .......................................................................... 35
3.14 PCR PRODUCT PURIFICATION AND SEQUENCING REACTION ............. 35
3.15 CYCLE SEQUENCING OF PCR PRODUCTS ............................................. 36
3.16 PURIFICATION OF SEQUENCED PRODUCTS ........................................... 37
3.17 SEQUENCE DATA ANALYSES ................................................................... 38
3.18 DATA ANALYSIS ......................................................................................... 39
LIST OF FIGURES

Figure 1: Structure of RSV Source ............................................................................. 7
Figure 2: G-Protein Structure source ........................................................................... 9
Figure 3: Structure of Human Metapneumovirus (HMPV) ........................................ 19
Figure 4: HMPV Genome ............................................................................................ 20
Figure 5: Frequency of clinical symptoms of children with ALRTI ............................. 45
Figure 6: Monthly distribution of RSVA, RSVB and HMPV over the study period ...... 48
Figure 7: Prevalence of RSVA, RSVB and HMPV in children with ALRI ................. 49
Figure 8: An electrophoregram (1.5% agarose gel in 1X TAE buffer) of RSV A amplification. ........................................................................................................ 51
Figure 9: An electrophoregram of amplified RSV B .................................................. 52
Figure 10: Gel electrophoresis of HMPV .................................................................. 53
Figure 11: Neighbour joining trees representing phylogenetic analysis of RSV genotypes isolated in Ghana between 2015 and 2016 .................................................. 55
Figure 12: Graphic view of nucleotide changes between RSV-A study samples and RSV-A ON1 reference genotypes within the G-gene .................................................. 56
Figure 13: Amino acid changes between RSV-A study samples and RSV-A ON1 reference genotype ................................................................. 57
Figure 14. Graphic view of nucleotide changes between RSV-B study samples and RSV-B BAIX reference genotypes ................................................................. 58
Figure 15: Amino acid changes between RSV-B study samples and RSV-B IX reference genotype .......................................................................................... 59
LIST OF TABLES

Table 1: Conventional PCR mix ........................................................................................................... 32
Table 2: Conventional PCR amplification protocol ............................................................................. 33
Table 3: Details of oligonucleotide used to amplify G and F Genes RSV and HMPV using conventional PCR .................................................................................................................. 34
Table 4: Sequencing reaction .................................................................................................................. 37
Table 5: Cycle sequencing conditions .................................................................................................. 37
Table 6: Demographic characteristics of participants with respect to age groups, gender, educational status and patient category ........................................................................................................ 41
Table 7: Association of clinical diagnosis with RSVA, RSVB and HMPV infections .... 42
Table 8: Demographic characteristics of parents’ or guardians with child’s results ........ 43
Table 9: Duration of stay in hospitals for RSVA, RSVB & HMPV patients .............................. 46
Table 10: The association of RSV and HMPV infection on breastfeeding .............................. 47
Table 11: Risk factor associations with RSVA, RSVB and HMPV infections among children under five years ................................................................................................................... 50
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALRTI</td>
<td>Acute lower respiratory tract infection</td>
</tr>
<tr>
<td>ALRI</td>
<td>Acute lower respiratory infection</td>
</tr>
<tr>
<td>ADVs</td>
<td>Adenoviruses</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>dNTPs</td>
<td>Deoxynucleoside triphosphates</td>
</tr>
<tr>
<td>Env</td>
<td>Envelope</td>
</tr>
<tr>
<td>HMPV</td>
<td>Human Metapneumovirus</td>
</tr>
<tr>
<td>MEGA</td>
<td>Molecular Evolutionary Genetics Analysis</td>
</tr>
<tr>
<td>MI</td>
<td>Milliliter</td>
</tr>
<tr>
<td>NS</td>
<td>Nasal swab</td>
</tr>
<tr>
<td>NM</td>
<td>Nanomoles</td>
</tr>
<tr>
<td>Nt</td>
<td>Nucleotide</td>
</tr>
<tr>
<td>RSV</td>
<td>Respiratory syncytial virus</td>
</tr>
<tr>
<td>RTI</td>
<td>Respiratory tract infection</td>
</tr>
<tr>
<td>RNP</td>
<td>Ribonucleoprotein</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PIVs</td>
<td>parainfluenza viruses</td>
</tr>
<tr>
<td>PMLCH</td>
<td>Princess Marie Louise Children’s Hospital</td>
</tr>
<tr>
<td>VR2</td>
<td>Second variable region</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
</tbody>
</table>
ABSTRACT

Acute lower respiratory tract infection (ALRTI) in children under five years is predominantly cause by Respiratory syncytial virus (RSV) but in recent times Human Metapneumovirus (HMPV) has also been implicated in ALRTI. In Ghana, the few studies on ALRTI have focused on the prevalence of RSV and the viral etiological agents causing acute bronchiolitis, bronchopneumonia and pneumonia.

This cross sectional study sought to detect, characterize and genotype respiratory syncytial virus and human metapneumovirus in children less than 5 years old presenting with ALRTIs infection at the Princess Marie Louis Children's Hospital in Accra, Ghana. Nasopharyngeal swabs were collected from September 2015 to November 2016 from 176 hospitalized young children below 5 years old for the detection of the two viral agents. A standardized questionnaire was used to obtain demographic data and medical record. Ribonucleic acid was extracted from the nasopharyngeal swaps using the QIAamp viral RNA kit (QIAGEN, USA) and cDNA syntheses performed by RT-PCR using random hexamer primers. All samples were analyzed using conventional PCR method for genotyping. Positive amplicons for RSV were sequenced, and analyzed for the different genotypes. The results were analyzed using SPSS version 22 and Stata statistical package version 13.

RSV and HMPV prevalence among the sampled subjects were 11.4% and 1.7% respectively. Of the RSV positives, 8/176 (4.5%) were RSV-A and 12/176 (6.8%) were RSV-B. The highest prevalence was observed in children less than 12 months old. No co-infection was observed in this study. Phylogenetic analysis of the second
hypervariable region of the G-gene of the RSV revealed that all RSV group A viruses belonged to the "novel" ON1 genotype containing the 72-nucleotide duplication and RSV group B viruses belong to the BA IX genotype.

RSV pathogen still remains a dominant viral etiological agent in acute lower respiratory tract infections among children below five years old.
CHAPTER ONE

1.0 INTRODUCTION

1.1 BACKGROUND

Viruses have been named as one of the etiological agents of acute lower respiratory tract infections (ALRTI). The most common viral causative agents are respiratory syncytial virus (RSV), parainfluenza viruses (PIVs), adenoviruses (ADVs), human rhinoviruses (HRVs), human coronaviruses (HCoVs) and influenza viruses (Macedo et al., 2003). Several other newly emerging viruses which cause lower respiratory infections have been recognized by advanced molecular techniques and one of such viruses is the Human metapneumovirus (HMPV) which has also been implicated as causative agents of ALRTI (Jain et al., 2014).

Following its discovery in 2001 in the Netherlands, infection caused by HMPV has increased worldwide and has proven to be the cause of both upper and lower respiratory tract infections in young children (Ramirez et al., 2014; Williams et al., 2004). RSV and HMPV have been identified as the prominent among the viruses associated to be a leading cause of childhood lower respiratory tract infection (LRTI) with severe clinical episodes (Jroundi et al, 2016; Manoh et al., 2007; von Linstow et al., 2004).

Both viruses belong to the same family Paramyxovirus, genus Pneumovirinae for RSV, and metapneumovirinae for HMPV. They are enveloped and single stranded
negative sense RNA virus. Phylogenetic analysis has revealed two sub- groups each as A and B (Gimferrer et al., 2015; Kahn, 2006).

HMPV can be subdivided into their genetic lineages A1 and A2 and B1 and B2 (Mohammed, 2014). Currently, there are over 13 genotypes for RSV group A and 23 genotypes of RSV group B (Arnott et al., 2011; Baek et al., 2012; Dapat et al., 2010; Eshaghi et al., 2012).

Both HMPV and RSV infect human populations and produce similar symptoms, although symptoms from the former are less severe than the latter. Clinical manifestations of symptomatic HMPV are also indistinguishable from those of RSV and both can cause severe disease often requiring hospitalization (Arnott et al., 2013; Cilla et al., 2009). A study in the United Kingdom demonstrated that HMPV and RSV co infection conferred an increased risk of admission to the paediatric intensive care unit (Semple et al., 2005). Some studies have also recorded both high and low incidence of HMPV/RSV co-infections owing to the fact that annual epidemics of both viruses partially overlap especially in temperate and tropical climates with a co infection rate of less than 10% (Al-sonbolí et al., 2006; Esper et al., 2004; König, König, & Arnold, 2004; Viazo et al., 2003).

Globally, RSV remains the main etiological agent that causes childhood lower respiratory tract infection (LRTI). This LRTI is associated with bronchopneumonia, pneumonia and bronchiolitis in children below 5 years (Lamarão et al., 2012; Munywoki et al., 2013; Ramirez et al., 2014). It was reported in 2010 that approximately 120.4 million episodes of ALRI cases of which 14.1 million were cases
of severe ALRI infections that occur in children below 5 years of age and about 1.4 million pneumonia deaths occur in this age group (Rudan, et al., 2008; Shi et al., 2015). African countries like Ghana, Nigeria, Gambia, Senegal, Chad, Cameroon, Burkino Faso and Mali have also shown an incidence rate of 15-21% in children under 5 years with ALRIs (Adiku, et al., 2015; Robertson et al., 2004). Ghana recorded ALRI cases such as bronchopneumonia, and pneumonia as some of the main reasons for hospitalizations and death among young children (WHO, 2016).

Generally, respiratory tract infections can be described as upper or lower. The upper respiratory infections affect the nose, throat and sinus, and the lower respiratory tract infection (LRTI) affects the airways and lungs (Macedo et al., 2003). Clinical presentations are similar in both developed and underdeveloped world, with mortality rates higher in developing countries (Broor et al., 2007).

The main symptom of ALRTI is characterized by severe cough associated with fever and mucus production. In addition, complications of ALRTI sometimes presents as bronchopneumonia, pneumonia and bronchiolitis (Popow-kraupp & Aberle, 2011). Bronchopneumonia and pneumonia are some of the main reasons for hospitalization and death among young children with ALRI infection in Ghana. The World Health Organization (WHO) in 2013 reported 13% deaths among children with ALRI (WHO, 2016).

However, there are some attributing factors influencing the incidence of respiratory tract infections. These include absence of exclusive breast feeding, age, exposition to environmental and passive smoke, malnourishment, overcrowding and vitamin A
insufficiency. Others may include poor socio-economic status of parents, parasitic infection, low birth weight and lack of RSV and HMPV vaccines (Calegari et al, 2005; Koch et al., 2003; Othman et al., 2016).

1.2 PROBLEM STATEMENT

Acute Lower Respiratory Tract Infections is one of the most common infections in young children worldwide (van den Hoogen et al., 2003). It has been estimated by WHO that complications arising from acute respiratory tract infections (mainly pneumonia), kills about 1.9 million children annually with a huge proportion of deaths (70%) occurring in Southeast Asia and Africa (Zhang et al., 2014). Though the burden of ALRTI is highest in Africa relatively, few studies have been carried out to know the aetiological agents. This poses a major hindrance to the effective surveillance and control of ALRTI in Africa.

In Ghana, few studies have determined the viral etiological agents causing ALRTIs. The majority of such studies have so far focused on the prevalence of RSV characterization and a few other viral agents such as parainfluenza and influenza viruses (Annan et al., 2016; Adiku et al., 2015; Kwofie et al., 2012; Obodai et al., 2014). Currently, there is no published data on the prevalence of HMPV as well as genotyping of RSV and cases of HMPV and RSV co-infection in Ghana. Clinical symptoms of HMPV and RSV are indistinguishable and both can cause severe disease often requiring hospitalization (Arnott et al., 2013; Cilla et al., 2009). This can lead to the abuse of antibiotics when the exact aetiology of ALRI is not known. It has been
established that RSV and HMPV co-infection may increase the severity of ALRTI cases in children. However, the contribution of the two viruses to ALRI cases seen is not clear.

1.3 JUSTIFICATION

Ghana is a tropical country, which records high prevalence of ALRTI cases all year round. Acute lower respiratory tract infection is a major public health concern which places great burden on families and society. This study will generate relevant data concerning RSV and HMPV and their co-infection and highlight their significant role in causing ALRTI cases in Ghana. This information will therefore be of immense value to our public health authorities to develop effective interventions in Ghana to help control the burden and also generate data on seasonal prevalence of both HMPV and RSV. The data generated will also enable physicians handling ALRTI cases consider HMPV/RSV infections as possible risk factors when formulating decisions pertaining to developing management options. The study may help ascertain whether the duration of hospitalization of ALRTI cases prolong with the co-infections of these two viral agents.

1.4 AIM

The aim of this study was to detect and characterize RSV and HMPVs from children below five years old who have been clinically diagnosed with ALRTI at the Princess Marie Louise hospital in Accra.
1.4.1 SPECIFIC OBJECTIVES

- To determine the role of RSV and HMPV in ALRTI
- To determine clinical characteristics associated with RSV and HMPV
- To determine the risk factors associated with RSV and HMPV in ALRTI cases.
CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 HISTORY AND STRUCTURE OF RESPIRATORY SYNCTIAL VIRUS

In the late 1950's to early 1960s, Robert M. Chanock who first recognize and isolated the characterized virus as shown in Figure 1 observed that a fever characteristic of catarrh was observed in infants and was similar to a virus serologically identical to infect the respiratory systems of Chimpanzees. He then proposed a name 'Human Respiratory Syncytial Virus' (HRSV) due to the cellular changes that occur within the lungs and airways of an infected individual following an infection (Craig, 2003). RSV belongs to the viral family *Paramyxoviridae*, subfamily *Pneumovirinae* within the genus *Pneumovirus*.

![Respiratory Syncytial Virus](image)

**Figure 1:** Structure of RSV Source (Shutterstock, 2017).
The single-stranded, enveloped, nonsegmented, negative-sense viral particle of 15.2 Kb is asymmetrically spherical in shape. The RSV genome has a diameter of about 150nm that encodes 10 proteins, and codes for 11 genes of which two are non-structural (Hall, 2001).

The helical viral ribonucleoprotein (RNP) complex consists of the RNA genome which is encapsidated by the nucleoprotein (N), phosphoprotein (P) and RNA-dependent RNA polymerase (L) as well as the M2-1 protein (Cervantes-Ortiz, Natalia Zamorano, & Grandvaux, 2016; Mccracken et al., 2014). The viral genome also encodes the structural matrix protein (M), Matrix M2-2 protein. The two non-structural proteins, NS1 and NS2 and three transmembrane surface proteins; namely the small hydrophobic protein (SH), attachment glycoprotein (G) and the fusion protein (F) which is immunogenic in nature and elicits antibodies with neutralizing capabilities but the G does not significantly stimulate cytotoxic T-lymphocyte responses (Arnott et al., 2011; Sullender, 2000). The G protein is responsible for viral attachment to cells and has greater variability than that of the other proteins both between and within the major antigenic groups of RSV.

However, it is said to lack the neuraminidase or hemagglutinin activity of the other Paramyxovirus attachment protein. A type I transmembrane glycoprotein known as the F protein, arise from the cleavage of an N-terminal signal sequence and has a transmembrane anchor located close to the C-terminus. Following synthesis, the F protein is modified by incorporating N-linked sugars after which it is cleaved to two subunits; F1 and F2, linked by disulphide bonds. After synthesis and modification by the addition of N-linked sugars, is cleaved to the two subunits, F1 and F2, linked by
disulphide bonds. The F glycoprotein promotes fusion and mediates virus entry into cell membranes. The infected cell and that of other neighbouring cells then promote "syncytial formation" (Mcnamara & Smyth, 2002; Sullender, 2000).

2.2 MOLECULAR EPIDEMIOLOGY OF RSV

RSV is grouped into two phylogenetic groups A and B based on their genetic diversity and antigenic differences that exist in the attachment (G) glycoprotein (Mufson et al., 1985). Each group can further be subdivided into genotypes by nucleotide sequence variability. The G protein (Figure 2), a type II surface glycoprotein is heavily glycosylated with N and O-linked sugars which consist of three domains namely; an ectodomain, a transmembrane domain and a cytoplasmic domain of about three hundred amino acids in length (Schnitzler, 2014). The G protein’s ectodomain is rich in threonine and serine residues potentially functioning as sites for accepting O-linked sugars (Reiche & Schweiger, 2009).

Figure 2: G-Protein Structure source: (Sullender, 2000). 'A' is a primary amino acid structure of the G Protein of an A2 Isolate. 'B' is the amino acid region that is naturally conserved among all human RSV G-proteins. This is indicated by amino acid 164 – 176. 'C' is the linear region of the G protein.
RSV has two hypervariable regions (HVR) and its C-terminal region (that's the second hypervariable region) mostly accounts for strain-specific epitopes (Shobugawa et al., 2009; Cane & Pringle, 1995). Furthermore, it has been shown that the differences found within the different RSV types are present in the ectodomain mainly of the G protein, which share 44% amino acid sequence only to the identity between the two groups, as compared to 83% identity in the cytoplasmic and trans-membrane domains (Hirsh et al., 2014; Reiche & Schweiger, 2009). The RSV of both groups simultaneously circulate or co-circulate with one group being predominate during epidemics.

Presently, there are over 13 genotypes of RSV A subgroups based on nucleotide sequence analysis. GA1 – GA7, SAA1, SAA2, NA1, NA2, NA3, NA4, CB-A and ON1 being a "novel" genotype with a 72-nt duplication firstly reported in Ontario, Canada in 2010 (Eshaghi et al. 2010). RSV subgroup B has currently 23 groups namely GB1 – GB4, SAB1 – SAB3 and BA 1-12, URU1, URU2, BA I- XIII and THB (Arnott et al., 2011; Baek et al., 2012; Dapat et al., 2010; Eshaghi et al., 2012; Parveen et al., 2006; Shobugawa et al., 2009).

Over the years, RSV strains have shown to accumulate amino acids substitutions, suggesting antigenic drift (Cane & Pringle, 1995). In 1999, an RSV-B new genotype, BA emerged in Buenos Aires, Argentina, containing a 60-nucleotide (nt) duplication in the second hypervariable region (VR2) of the G gene and had spread widely and replaced all previously described RSV-B genotypes (Otieno et al., 2017). Therefore, there is a possibility of a selection advantage resulting from the nucleotide duplication of the ON1 genotype owing to the fact that there is a steady spread of the BA
genotype which is the currently dominating genotype (Schnitzler, 2014). In Ghana, previous epidemiology and genetic studies of circulating RSV strains revealed that both RSV-A and RSV-B co-circulate among the study population, although Group B viruses were predominant (Obodai et al., 2014).

2.3 RSV TRANSMISSION

RSV is easily transmitted and spread from an infected individual by respiratory secretions on hands, or formites. Direct contacts with large aerosols or droplets usually have a higher impact than airborne transmission. The nose and eyes are the most sensitive inoculation areas thus proper hand washing techniques and keeping the hands away from these infected areas decrease transmission. For several hours, RSV can survive on surfaces and non-porous surface for about 12 hours and over 30 minutes on the hands. The incubation period is between 3-5 days and viral shedding usually occurs for 3-8 days but could persist in the body for as long as 4 weeks in younger children and immunocompromised patients (Eiland, 2009; Light, 2012).

2.4 PATHOGENESIS OF RESPIRATORY SYNCTIAL VIRUS

RSV infection begins with replication in the nasopharynx targeting the ciliated airways of the epithelial cells. The virus binds and spreads to the small bronchiolar epithelium lining the small airways of the lungs, and necrosis of the cell occurs. Hypersecretion of mucus occurs, and infiltration of the rounded cells causes oedema
in the surrounding submucosa (Eiland., 2009) As a result, the small airways of the lumina becomes obstructed, there is an increase in the peripheral areas due to air trapping and atelectasis occurs when the trapped air is absorbed. Chemokines, interleukins and leukotrienes are released which causes tissue damage and necrosis. In a lower respiratory tract infection, the possible mechanism for the spread of RSV is through infection of the macrophages, with migration to the lower airways (Domachowske & Rosenberg, 1999). Hence, an increase in mucus production, edema and eventual necrosis and regeneration of these epithelial cells. This therefore leads to air trapping and obstruction of the airway as well as increased in airways resistance. Epithelium recovery starts in the first week of the disease but the ciliated cells do not recover until weeks later (Dawson-Caswell & Muncie, 2011; Sullender, 2000).

2.5 CLINICAL PRESENTATIONS

Nearly all children may have evidence of serologic RSV infection by 2 years of age. RSV infection primarily is often not asymptomatic and the peak period of disease severity due to annual hospitalization rates is 17 per 1000 children under 6 months of age and 3 per 1000 children under 5 years of age during the first few months of life (Popow-kraupp & Aberle, 2011). Some clinical manifestations following RSV infection is children experience mild like- cold-symptoms with nasal congestion. However, due to the lack of internationally agreed-upon definitions for a case definitions as having an ALRTI, a child should have a cough or difficulty in breathing and must have one or more of the following: fast breathing, lower chest wall in
drawing, stridor, wheezing or apnoea. Fast breathing was defined as > 60 breaths per minute in children aged < 2 months 50 per minute in children aged 2–11 months, and as > 40 per minute in children aged 12–59 months and require hospitalization. Normally cough develops in the first few days and becomes prominent as infection proceeds. It may however remain on for weeks due to the slow recovery of the ciliated cells and about 50% of infected children experience this cough (Munywoki et al., 2013; Robertson et al., 2004).

Secondly, there is increase in respiratory rate, apnoea, wheezing, poor feeding and respiratory distress (Tregoning, 2010). Retraction irritability or lethargy and lower intercostal muscles herald involvement of the lower respiratory tract with or without fever (Popow-kraupp & Aberle, 2011).

Other symptoms which may be accompanied by clinical signs rarely is cyanosis; auscultation of the chest wheeze, reduced breath sounds due to air trapping and peripheral hyper- inflation of the lung. Extrapulmonary manifestations of ALRTI have also been described for RSV infection although it is rarely observed. This includes, cardiac failure, seizures, hepatitis and cardiac arrhythmias (Tregoning, 2010). In most infants, the duration of symptoms is between seven to twenty one days and hospitalization rates of 25% to 40% develop symptoms of pneumonia and bronchiolitis during their primary RSV infection, whilst some need ventilatory support (Popow-kraupp & Aberle, 2011).
Generally, there is a variation between clinical presentation of patients and their clinical evolution pattern, but they are largely related to pneumonia and bronchiolitis in severe cases like respiratory failure (Riccetto et al., 2009).

2.6 BURDEN OF DISEASE ASSOCIATED WITH RSV

Mortality data suggest globally that RSV is a significant cause of deaths and hospitalizations in infants under 5 years. In the United States alone, it is estimated that 51,000–82,000 infants are hospitalized annually with RSV bronchiolitis, generating an economic burden of $365 million to $585 million (Papenburg et al., 2012).

Furthermore, the Global Burden of Diseases, Injuries, and Risk Factors Study 2010 (GBD 2010) estimated that 2.8 million deaths were due to lower respiratory infections globally (5.3% of the total deaths) and the incidence of ALRI in children under 5 years per child-year has been estimated as 0.22 episodes and 11.5% cases lead to severe episodes in developing countries in 2010 in which most cases occurred in Nigeria, China, Bangladesh, India, Pakistan and Indonesia (Feng et al., 2014). Similarly from a study in Kenya, it has been reported that among hospitalized infants, the incidence of ALRTI ranged from 4.8% in the first year of life to 0.1% among older children (Berkley et al., 2010)
2.7 LABORATORY DIAGNOSIS

Laboratory methods currently available for the detection and confirmation of RSV include virus isolation/culture and serology which has been the gold standards for over two decades (Khalilzadeh et al., 2010). Viral antigen detection is by direct or indirect immunofluorescent (IF) staining (DFA/IFA) or by enzyme-linked immunosorbent assays (ELISAs), and the detection of nucleic acids by amplification assays, of which the nucleic acid amplification technique is largely being used, is the reverse transcription polymerase chain reaction (RT-PCR) which allows rapid detection of many viruses concurrently (Popow-kraupp & Aberle, 2011).

Numerous emerging molecular techniques are gaining prospects of being applied not only in diagnosing respiratory viral infections but also for monitoring purposes. This includes quantum dots, loop-mediated isothermal amplification (for direct detection of nucleic acids), multiplex ligation-dependent probe amplification, solid and suspension microarrays, target-enriched multiplexing amplification, pyrosequencing, amplification using arbitrary primer and mass spectrometry (Akhter & Johani, 2009; Wu & Tang, 2009). A lot of these assays have been made available commercially to offer multiplex amplification and some promote identification panels of these respiratory viral pathogens which further enhances the rapid diagnosis of respiratory viral infections (Akhter & Johani, 2009).
2.8 RISK FACTORS ASSOCIATED WITH RSV INFECTIONS

The associated risk factors for RSV severe infection have been thoroughly characterized such as infants birth season, exposition to air smoke and pollution, age when infected, male gender, low socioeconomic status of parents or caregivers, increased exposure to the infectious agent, day care attendance, hospitalization, malnutrition, low birth weight and nonexclusive breast feeding in the first 6 months after birth (Boloursaz et al., 2013). Others also include indoor crowding and lack of immunization in children under one year of age, mother’s education experience as a caregiver, cold weather, vitamin A deficiency, and factors affecting the lungs (bronchopulmonary dysplasia) and immune immunodeficiency disorder such as HIV/AIDS (Koch et al., 2003; Light, 2012; Macedo et al., 2003).

2.9 SEASONALITY

RSV has seasonal distribution patterns as viruses circulate in winter and spring months with recurrent epidemics occurring in winter. (Red & Medical, 2012). In temperate countries for instance, RSV outbreaks occur mostly during winter and fall with a well-defined seasonal pattern (Calegari et al., 2005). In the temperate, most communities have defined seasons between 3 to 5 months usually starting in the northern hemisphere in October or November and continue through to March.

In subtropical and tropical countries, the rates of RSV infections are greater in winter with considerable regional variations. Epidemiological studies on RSV in the
southeast region of Brazil showed that the infection by RSV starts in April with peaks in May and June. In Ghana, RSV infection peaks during the rainy season in July through to February (Annan et al., 2016; Adiku et al., 2015; ;Obodai et al., 2014).

2.10 TREATMENT AND MANAGEMENT OF RSV INFECTION

There are no specific treatments for RSV infection. Treatment is primarily supportive although a number of regimen have been tried including antiviral agents, bronchodilators and nasal suctioning. None of these treatments has had a significant impact on the symptoms or course of this disease. The management strategies are maintenance of hydration and administration of oxygen (Brown & Lidbury, 2008).

Ribavirin, an antiviral drug has clinically been proven in vitro to be effective against RSV and is licensed for use by inhalation in patients severely suffering from lower respiratory tract infections such as bronchiolitis (Cane, 2001; Tregoning, 2010). Prophylaxis with a specific anti-RSV monoclonal antibody (palivizumab), an FDA approved monoclonal antibody for RSV is recommended in high-risk cases (Dawson-Caswell & Muncie, 2011). Its targets the F (fusion) glycoprotein and prevent viral entry into host cells. Some studies have also demonstrated its use to reduces rates of hospitalization by 55%, and duration of hospital stay by 42% and the number of days with need of oxygen by 40% (Eiland, 2009; Tregoning, 2010).

In 2015, the WHO Product Development for Vaccines Advisory Committee (PDVAC), stated that very few different vaccines are being developed and being tested for phase 1–3 trials so there is an anticipation that these may become available
to the health sectors in the short term and help reduce the disease burden in developed and under developed countries (Giersing et al., 2016).

2.11 HISTORY AND STRUCTURE OF HUMAN METAPNEUMOVIRUS (HMPV)

The isolation and characterization of a "novel" Paramyxovirus named Human metapneumovirus (HMPV) was initially done from twenty eight children formerly virus-negative nasopharyngeal aspirates and suffering from various respiratory tract infection in the Netherlands (van den Hoogen et al., 2001). He and his colleagues observed that, infants that had fever characteristic of catarrh were similar to a virus serologically identical and closely related to RSV. He then termed the new virus HMPV (Figure 3). Since then HMPV has been detected worldwide (Greensill et al., 2003; Heikkinen et al., 2008; König et al., 2004; Lazar et al., 2004; Peret et al., 2002)
Figure 3: Structure of Human Metapneumovirus (HMPV) Source: (Mackay, 2017)

Human metapneumovirus belongs to the Paramyxoviridae family, genus Metapneumovirus, subfamily Pneumovirinae within the genus Pneumovirus (Chung et al., 2006). With about 13.3kb size (Fig 4), the RNA genome of HMPV has eight genes encoding 9 different proteins. Identical to this virus in the order is the avian pneumovirus (AMPV) (Haas et al., 2013; Vinomarlini et al., 2014). The single-stranded viral particle is enveloped, nonsegmented, negative-sense and spherical in shape and measures about 150-600nm (Ramil & Domi, 2010; Vinomarlini et al., 2014). The genome consists of about 13,350 nucleotides comprising of a Nucleoprotein (N), phosphoprotein (P) matrix protein (M2-1 and M2-2) and an open reading frame (ORF) that encodes three viral glycoproteins namely, the F (fusion), G
SH (short hydrophobic) proteins and an RNA-dependant RNA polymerase. Of these proteins, the F protein is highly the conserved region between HMPV and RSV by 33% amino acids and also codes for a class 1 viral fusion entry which mediates virus entry via attachment and fusion into the host cells membranes (Mohamed et al., 2014). The F is said also to be immunogenic and induces protective antibodies. The PCR target the polymerase (L) regions and nucleoprotein (N) and the conserved nature of all the HMPV strains (Vinomarlini et al., 2014). The two other surface glycoproteins, G and SH are different from other paramyxoviridae and has clearly been shown to be weak or negligibly immunogenic (Adamson, Thammawat, Muchondo, Sadlon, & Gordon, 2012). HMPV however, is said to be deficient with two non structural genes "NS-1 and NS-2" at the 3’ end of the genome (Kahn, 2006).

![HMPV Genome](image)

**Figure 4:** HMPV Genome. Source: (Wen & Williams, 2015)

### 2.12 MOLECULAR EPIDEMIOLOGY OF HMPV

Since its initial recognition in 2001, the epidemiology of HMPV has been described all over the world in infants diagnosed with ALTRI cases and by the age of 5 years approximately 70% of all children develop antibodies to HMPV infection.
Worldwide, the incidence rate has shown a wide range infection for about 1.5% to 17\% (Owor \textit{et al.}, 2016) using either cell culture, serology or mostly PCR (Mahalingam \textit{et al.}, 2006; Milder & Arnold, 2009).

Two HMPV genotypes have been known as A and B, with each group subdividing further into two more lineages: that is A1, A2, and B1, B2. In 2006, a novel sub-lineage within the viral subgroup A2 was identified, which was named A2a and A2b (Broor \textit{et al.}, 2008; Huck \textit{et al.}, 2007; Ramil & Domi, 2010). The amino acid uniqueness sequence of the F proteins of these viruses belonging to lineages A and B is 95 to 97\% and are only 30 to 35\% in contrast identity to G protein sequences (Schildgen \textit{et al.}, 2011).

\textbf{2.13 TRANSMISSION}

HMPV route of transmission is comparable to the pattern described for RSV. The infection occurs via respiratory droplets as occurs with RSV through an infected person to another through secretions from sneezing and coughing. Close contact, such as shaking of hands and touching of surfaces that have the viruses on them, then touching the eyes, mouth and nose (Haas, Thijsen, Elden, & Heemstra, 2013; Mahalingam \textit{et al.}, 2006).
2.14 PATHOGENESIS OF HMPV

HMPV replicates extensively in the upper and lower respiratory tracts. For members belonging to the subfamily pneumovirinae (including genus Pneumovirus and metapneumovirus), the mode of attachment involves the interaction of the G protein on the host cell surface (Levine, Klaiber-franco, & Paradiso, 1987). To date, some studies also suggest that one more than one heparin-like molecules and cellular glycosaminoglycans are involved in the virus attachment and entry. Following the G-protein attachment, the F glycoprotein promotes fusion between cell membranes and the virus envelope. To become functional, the F protein is cleaved by cellular proteases and the hydrophobic amino terminal region of the F1 component promotes the fusion process, which then introduces internal components of the virion into the cytoplasm of the host cell where the rest of the infectious cycle takes place. In an experiment to demonstrate HMPV pathogenesis in lung infection in small animal models, Yim and his colleagues used two animals namely the BALB/c mice and cotton rats and their results demonstrated that both animals efficiently support viral replication (Yim et al., 2005).

2.15 CLINICAL PRESENTATIONS OF HMPV

Clinical symptoms of HMPV infection bear resemblance to those caused by respiratory syncytial virus (Heikkinen et al., 2008) and the manifestations of affected children range from mild upper respiratory tract disease like cough, rhinorrhea, croup
to severe lower respiratory disease such as pneumonia, bronchiolitis, and which may include cough, fever, nasal congestion, erythema, myalgia, and in severe cases, may have respiratory difficulty, dysphonia, stridor, wheeze and respiratory failure which require hospitalization (Kahn, 2006; von Linstow et al., 2004).

2.16 SEASONALITY

The circulation of HMPV is worldwide and varies between populations. Seasonal distribution is mainly in winter to early spring in the temperate (Horthongkham et al., 2014) whereas it starts from spring to summer in tropical regions. Sometimes the same strain may spread in different locations and at different times. The spread is still complex and unclear. However in Kenya, prevalence of HMPV has been recorded around June to July in the west and in November to December in refugee camps in the north east and west of the Country (Owor et al., 2016).

2.17 LABORATORY DIAGNOSIS OF HMPV

In cell culture, HMPV grows poorly because sensitivity is due to low detections in the secretions of the respiratory tract and relatively difficult to isolate (Broor et al., 2008; Kahn, 2006). Secondly, its replication in vitro is constrained by a number of cell lines which often requires trypsin supplementation of the medium for propagation. In a recent study using 19 different cell lines to grow HMPV, it has been shown that the most suitable cell lines for the growth were a human Chang conjunctiva cell line
Numerous genes for HMPV have been the prime targets for genomic amplification. Currently, the conventional and real time PCR assays are the commonest methods used to detect HMPV and other respiratory viruses. For the real time assays, more genes have been targeted to detect the virus for both genotypes and all known lineages (Maertzdorf et al., 2004). The Immunofluorescence assays which are also virus-specific antibodies are also employed in their detection (Ricetto et al., 2009).

2.18 DISEASE BURDEN ASSOCIATED WITH HMPV

HMPV and RSV Hospitalization are high in infants. The annual hospitalization rates in U.S among those aged between 0–5 months were recently estimated as 4.9 cases/1000 children and 17 cases/1000 children respectively and an estimated 27,000 were HMPV-related hospitalizations that occurred among preschool children per year (Papenburg et al., 2012).

2.19 TREATMENT AND MANAGEMENT OF HMPV

There are no specific antiviral therapies for HMPV therefore medical care is primarily supportive. However, few trial vaccines have been tested in animal models. A recombinant live human parainfluenza virus that contains the HMPV F gene has been
shown to induce HMPV-specific antibodies (Kahn, 2006). Presently, live attenuated
HMPV vaccines seem promising especially for infants and children (Ren et al., 2015).
CHAPTER THREE

3.0 METHODOLOGY

3.1 STUDY DESIGN

The study was a cross-sectional study among children less than five years with Acute Lower Respiratory Tract Infections (ALRTI).

3.2 STUDY SITE

The study participants were recruited from the Princess Marie Louise Children’s Hospital (PML) in the Greater Accra Region. The PML is a major children referral hospital within the Ashiedu Keteke sub metro of the Greater Accra region that provides medical care and offers reproductive and child health services to children, individuals and households in Accra and beyond. As of the period of this study, there were six permanent doctors, eighty-one nurses and two hundred and sixty five other paramedical staff. The medical service of PML consists of 74 beds, Emergency ward, an out-patient Department (OPD), Child Health (RCH), Laboratory unit/blood bank, X-Ray Unit, Dietetics and Environment Health Unit, Mothers’ Hostel, Disease Control Unit among others. Within the last decade, attendance to the P.M.L. Out-patient (OPD) has almost doubled from 45,000 in 1996 to nearly 73,000 per year.
3.3 RECRUITMENT OF SUBJECTS

Children below five years and clinically diagnosed of ALRTI at the study site were recruited for this study between September 2015 and November 2016. Demographic data and clinical information were obtained. A standardized questionnaire and medical record review which captured data on age, sex and clinical symptoms such as cough, fever, nasal discharge, fast breathing, difficulty in breathing, vomiting, diarrhoea, oxygen administration and length of hospitalization were recorded.

3.4 INCLUSION CRITERIA

Children below the age of five years who have been clinically diagnosed with ALRTI and consented to the study were included in the study.

3.5 EXCLUSION CRITERIA

The following were excluded from the study; children known with chronic lung disease, congenital heart abnormalities and asthma.

3.6 CASE DEFINITION OF ACUTE LOWER RESPIRATORY INFECTION (ALRTI)

Case definitions for a child having a ALRTI is defined as having a cough or difficulty in breathing and should have one or more of the following: fast breathing, lower chest
wall wheezing, in drawing, stridor, or apnoea. Fast breathing was defined as > 60 breaths per minute in children aged < 2 months, > 50 per minute in children aged 2–11 months, and as > 40 per minute in children aged 12–59 months (Hasan, Rhodes, & Thamthitiwat, 2014; Munywoki et al., 2013; Reed et al., 2012; Robertson et al., 2004; Wu et al., 2015)

3.7 SAMPLING SIZE

The formula for sample size determination by (Rose, Spinks, & Canhoto, 2014) was used to estimate the number of children needed for this study.

\[ n = \frac{t^2 \cdot p(1-p)}{m^2} \]

where

- \( n \) = sample size needed
- \( t \) = 95% confidence level (standard value of 1.96)
- \( m \) = margin of error at 5% (standard value of 0.05)
- \( p \) = prevalence estimate of ALRI infection based in Ghana in the project area (16%) (Adiku et al., 2015; Kwofie et al., 2012)

The minimum sample size required for the hospital study was estimated to be 207.

3.8 SAMPLING METHOD

A convenient simple random sampling method was used to recruit children into the study after parents or guardians consented to the study.
3.9 TOOLS AND TECHNIQUES FOR SAMPLE AND DATA COLLECTION

3.9.1 Questionnaire
A structured questionnaire as referenced in Appendix II was administered to participants to obtain demographic characteristics and possible risk factors that predispose them to HMPV and RSV infection.

3.9.2 Sample collection/Transportation /Storage
Nasopharyngeal specimens (NS) were collected from ALRTI children below five years whose guardians or parents had voluntarily given their consent. The nasal swabs which effectively dislodge and collects virus infected cells lining the nasopharynx were inserted in a depth of about 2 cm into the naris. (Becton Dickson (BD), USA). The Swabs was rotated gently to collect exfoliates of cells. They were then aseptically removed and inserted into a BD vial containing a transport medium of about 2ml. The shafts of the swabs were carefully broken by bending it against the vial wall evenly at a pre-scored line. The tube was then replaced with a cap, closely tight and labeled with appropriate patient identification numbers. The samples were then placed on ice and sent immediately to the Virology unit of the Noguchi Memorial Institute of Medical Research (NMIMR) where they were be kept at -80°C until they were processed.
3.10 LABORATORY INVESTIGATION/ PROCEDURE

3.10.1 Sample preparation (RNA extraction)

Ribonucleic acid (RNA) was extracted from the respiratory specimens according to the manufacturer’s instructions using the QIAamp viral RNA mini kit (User Manual, QIAamp viral RNA mini kit, 2014).

Briefly, approximately 140μl of the respiratory specimens were lysed under highly denatured conditions, in a volume of 560μl prepared buffer AVL (AVL+RNA carrier) which inactivate RNases and ensured intact viral isolation. This mix was pulse vortexed for 15 seconds and incubated at room temperature (15-25°C) for 10 minutes. An absolute ethanol of about 560μl was added to the mix and pulse-votexed for another 15 seconds. Then, the mixture solution of about 630μl was carefully aliquoted and transferred to the QIAamp Mini column provided in the kit. This was centrifuged at 8000rpm for 1minute and this procedure was repeated twice with the remaining mix in a new collection tube. The filtrate was discarded and a new column placed and a pipetted 500μl AW1 buffer added. This was again span at 8000rpm for another 1 minute. With a new column placed under a collection tube, 500μl of buffer AW2 added, and span at 14000rpm for 3 minutes. The filtrate again was discarded and span again for 1 minute. RNA was eluted with 60μl of Buffer AE in a clean 1.5ml microcentrifuge tube. The eluted RNA was stored in minus 30°C freezer until further analyses. A negative extraction control (sterile molecular grade water) was used in parallel to all procedures.
3.11 REVERSE TRANSCRIPTION (RT) OF VIRAL RNA

Complementary DNA (cDNA) synthesis was performed with 25μl of RNA extract in a 40μl mixture containing 200μM of each deoxynucleoside triphosphate (dNTPs), 5mM dithiothreitol, 20U RNasin, a 250nM of random hexamer primers (Invitrogen-Thermo Fisher Scientific GmbH, Schwerte, Germany), 100U Moloney murine leukemia virus reverse transcriptase and first-strand buffer containing 250mM Tris-HCl (pH 8.3), 37.5mM KCl, and 15mM MgCl₂. 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 Applied Biosytems 2720 thermocycler (Applied Biosystems, Foster City, CA).

3.12 CONVENTIONAL PCR

A traditional conventional PCR method was employed for the detection and amplification of RSV G gene and F gene for HMPV. Samples that were positive for RSV were further subjected for differentiation into A and B groups. Each reaction mixture contained PCR buffer (Invitrogen Germany), 5mM MgCl₂, 100μM dNTPs,(Qiagen) 250nM of each specific primer, 0.5-unit SuperScript (R)III one step RT- PCR System with platinum Taq DNA polymerase (invitrogen) and 5.0 μl of cDNA with a final volume of 50 μl. Semi nested PCR reaction was then carried out as described in Table 1 using 2μl of the first round PCR product. All PCRs were performed using the Applied Biosystems Instruments (ABI 2720) thermocycler. Table 2 shows the amplification cycling conditions. Amplification of the specific genes for
RSV and HMPV pathogens were carried out using published primers as shown in Table 3.

**Table 1: Conventional PCR mix**

<table>
<thead>
<tr>
<th>REAGENT</th>
<th>RSVA</th>
<th>RSV B</th>
<th>HMPV</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X Reaction Buffer</td>
<td>1x</td>
<td>1x</td>
<td>1x</td>
</tr>
<tr>
<td>Mg²⁺ (25mM)</td>
<td>2mM</td>
<td>2mM</td>
<td>3mM</td>
</tr>
<tr>
<td>DNTP (10mM)</td>
<td>200uM</td>
<td>200uM</td>
<td>100uM</td>
</tr>
<tr>
<td>Taq Polymerase(5U/ul)</td>
<td>0.5U/ul</td>
<td>0.5U/ul</td>
<td>0.5U/ul</td>
</tr>
<tr>
<td>External Primer 1 (25µM)</td>
<td>250nM (RSVA-G-F)</td>
<td>250 nM (RSVB-G-524-F)</td>
<td>250 nM (HMPV-3637-F)</td>
</tr>
<tr>
<td>Internal Primer 1 (25µM)</td>
<td>250 nM (RSVA-G-606-F)</td>
<td>250 nM (RSVB-G-603-F)</td>
<td>250 nM (HMPV-3637-F)</td>
</tr>
<tr>
<td>Internal Primer 2 (25µM)</td>
<td>250 nM (RSVA-F-22-R)</td>
<td>250 nM (RSV-F-22-R)</td>
<td>250 nM (HMPV-4164-R)</td>
</tr>
<tr>
<td>Template (cDNA) external</td>
<td>5 µl</td>
<td>5 µl</td>
<td>5 µl</td>
</tr>
<tr>
<td>Template (cDNA) internal</td>
<td>2 µl</td>
<td>2 µl</td>
<td>2 µl</td>
</tr>
<tr>
<td>Nuclease free water</td>
<td>Top up to 50</td>
<td>Top up to 50</td>
<td>Top up to 50</td>
</tr>
</tbody>
</table>
Table 2: Conventional PCR amplification protocol

<table>
<thead>
<tr>
<th>Assay</th>
<th>Initial Denaturation</th>
<th>Number of cycles</th>
<th>Denaturation Annealing</th>
<th>Elongation</th>
<th>Final Extension</th>
<th>Cool 4°C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Time (min)</td>
<td>Temp (°C)</td>
<td>Time (s)</td>
<td>Temp (°C)</td>
<td>Time (min)</td>
<td>Temp (°C)</td>
</tr>
<tr>
<td>External PCR</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RSV A</td>
<td>30</td>
<td>94</td>
<td>45</td>
<td>72</td>
<td>10</td>
<td>72</td>
</tr>
<tr>
<td>RSV B</td>
<td>30</td>
<td>94</td>
<td>45</td>
<td>53</td>
<td>10</td>
<td>72</td>
</tr>
<tr>
<td>HMPV</td>
<td>30</td>
<td>94</td>
<td>30</td>
<td>60</td>
<td>45</td>
<td>72</td>
</tr>
<tr>
<td>Semi/nested PCR</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RSV A</td>
<td>30</td>
<td>94</td>
<td>30</td>
<td>94</td>
<td>10</td>
<td>72</td>
</tr>
<tr>
<td>RSV B</td>
<td>30</td>
<td>94</td>
<td>30</td>
<td>94</td>
<td>10</td>
<td>72</td>
</tr>
<tr>
<td>HMPV</td>
<td>30</td>
<td>94</td>
<td>30</td>
<td>94</td>
<td>10</td>
<td>72</td>
</tr>
</tbody>
</table>
Table 3: Details of oligonucleotide used to amplify G and F Genes RSV and HMPV using conventional PCR

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Polarity</th>
<th>Sequence (5'-3')</th>
<th>GENE</th>
<th>Expected Band size</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>RSV A</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RSVA-G-F</td>
<td>Forward</td>
<td>AGTGTTCAACTTTGTACCCTGC</td>
<td>G</td>
<td>593bp</td>
<td>(Reiche &amp; Schweiger, 2009)</td>
</tr>
<tr>
<td>RSV-F-R</td>
<td>Reverse</td>
<td>CTGCACCTGCATGGATGGATGAT</td>
<td>F</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RSVA-G-606-F</td>
<td>Forward</td>
<td>AACACCACCAAGCCCAACA</td>
<td>G</td>
<td>500bp</td>
<td></td>
</tr>
<tr>
<td>RSV-F-22-R</td>
<td>Reverse</td>
<td>CAACTCCATTGTATTTGCC</td>
<td>F</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RSV B</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RSVB-G-524-F</td>
<td>Forward</td>
<td>TTGTTCCCTGTAGTATATGTG</td>
<td>G</td>
<td>509bp</td>
<td></td>
</tr>
<tr>
<td>RSV-F-55-R</td>
<td>Reverse</td>
<td>AGTTAGGAAGATGGCACTTGA</td>
<td>F</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RSVB-G-603-F</td>
<td>Forward</td>
<td>AAAACCAACCATAAACCCAC</td>
<td>G</td>
<td>397bp</td>
<td></td>
</tr>
<tr>
<td>RSV-F-22-R</td>
<td>Reverse</td>
<td>CAACTCCATTGTATTTGCC</td>
<td>F</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HMPV</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HMPV-3637-F</td>
<td>Forward</td>
<td>GTYAGCTTCAGTCAATTCAACA GAAG</td>
<td>F</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HMPV-4192-R1</td>
<td>Reverse</td>
<td>CAGTGCAACCATACTGATRGGA TG</td>
<td>F</td>
<td>555bp</td>
<td></td>
</tr>
<tr>
<td>HMPV-4192-R2</td>
<td>Reverse</td>
<td>TAGTGCAACCATACTGATRGGG TG</td>
<td>F</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HMPV-3637-F</td>
<td>Forward</td>
<td>GTYAGCTTCAGTCAATTCAACA GAAG</td>
<td>F</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HMPV-4164-R</td>
<td>Reverse</td>
<td>ICCTGTGCTRACTTTGGCATGGG</td>
<td>TF</td>
<td>527bp</td>
<td>F</td>
</tr>
</tbody>
</table>

(Mohamed et al., 2014)
3.13 GEL ELECTROPHORESIS

Gel electrophoresis was employed to determine the yield and size fractions of the PCR products. Five microlitres (5μl) each of the PCR products were subjected to gel electrophoreses on 1.5% agarose gel in 1X Tris-acetate-EDTA (TAE) buffer (Life technologies, Ambion®, USA) with ethidium bromide (Life technologies, Invitrogen™, USA) staining. Samples were diluted in 1.5μl of 6X DNA loading buffer and 5μl were applied to the respective gel wells. A Molecular weight marker of 100bp was co-electrophoresed together with the samples. Running of gels in 1X TAE buffer was done at 80V for 45 minutes. The PCR products were then visualized under ultraviolet trans-illumination using the Benchtop 3UVTM Transilluminator Imaging system (Cambridge, UK). Positive samples were expected to have an average product size of 593bp for RSVA and 509 for RSVB during the first round PCR as shown in figures 8 and 9. For second round PCR, a product size of 500bp and 397bp was expected for RSVA and RSVB respectively (figure not shown). HMPV positive first round products had the expected size of 555bp, and the second round product was 527bp.

3.14 PCR PRODUCT PURIFICATION AND SEQUENCING REACTION

For the positive nested PCR products, purification was carried out using QIAquick PCR purification system (Qiagen, Hilden, Germany). Procedures were performed according to manufacturer’s recommendations (QIAGEN GmbH 2006). Briefly, a 1:5
dilution was prepared by adding 15 μl of nested products to 75μl of binding buffer (PB) mixed and vortexed. The mixture was then transferred to a spin column and centrifuged for 1 minute at 13200 rpm in (Eppendorf microcentrifuge 5415D, USA). The columns were washed by adding 0.75 ml (750μl) washing buffer and centrifuging for 1 minute at 13200 rpm. Filtrate was discarded and the procedure repeated twice. The DNA was eluted with 30μl elution buffer by centrifugation at 13200 rpm for 1 minute in a clean 1.5ml microcentrifuge tube. The eluted DNA was stored at -20°C until use.

3.15 CYCLE SEQUENCING OF PCR PRODUCTS

Cycle sequencing was performed using the Big Dye terminator v3.1 Cycle Sequencing kit (Applied Biosystems, California, USA). Cycle sequencing reactions for each viral gene was set up in both the forward and the reverse directions with the primer pairs used in the semi-/nested PCR. The sequencing reaction and cycling conditions were as follows: A 10μl reaction mixture, consisting of 2μl each of 5X Big Dye Sequencing Buffer, Big Dye Terminator Mix, 2μM primer, 3.4μl nuclease-free water and 2 μl purified PCR product, was used for cycle sequencing. The cycling conditions as summarized in table 4 were 94°C for 2 minutes, 25 cycles of 94°C for 30 seconds, 50°C for 15 seconds and 60°C for 4 minutes.
Table 4: Sequencing reaction

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Big Dye Terminator mix</td>
<td>1</td>
</tr>
<tr>
<td>5x Big Dye Terminator Buffer</td>
<td>2</td>
</tr>
<tr>
<td>Template (purified PCR product)</td>
<td>2</td>
</tr>
<tr>
<td>Primer (2µM)</td>
<td>1.6</td>
</tr>
<tr>
<td>RNAse free H₂O</td>
<td>3.4</td>
</tr>
<tr>
<td>Total sequencing reaction volume</td>
<td>10</td>
</tr>
</tbody>
</table>

Table 5: Cycle sequencing conditions

<table>
<thead>
<tr>
<th>Programme</th>
<th>Time (s)</th>
<th>Temp (°C)</th>
<th>Number of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denaturing</td>
<td>4</td>
<td>94</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Denaturing</td>
<td>15</td>
<td>50</td>
<td>25</td>
</tr>
<tr>
<td>Annealing/Elongation</td>
<td>240</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>Cooling</td>
<td>∞</td>
<td>4</td>
<td>1</td>
</tr>
</tbody>
</table>

3.16 PURIFICATION OF SEQUENCED PRODUCTS

Purification of sequenced product was performed with the Agencourt CleanSEQ sequencing reaction clean-up system (Agencourt Bioscience, USA). Procedures were performed according to manufacturer’s recommendations (Agencourt Bioscience Corporation: Protocol 000600v031, 2006). Briefly, 5µl of magnetic particles (Agencourt CleanSEQ) were added to the cycle-sequenced products (10 µl) and 42µl
of 85% ethanol was added to the mixture. The mixture was placed on a magnetic plate (Agencourt SPRIPlate 96R ring magnetic plate) for 3 minutes at room temperature. After that, the liquid portion of the mixture was carefully taken out and discarded leaving the DNA bound to the beads on the wall of the tube. With the tube still on the magnetic plate, 100 µl of 85% ethanol was added to the tube for 3 minutes. The liquid was then carefully taken off leaving the DNA bound to the beads on the wall. The tube was left on the magnetic plate for 10 minutes for the ethanol to completely dry off. Forty microliters of nuclease-free water (Life technologies, Ambion, USA) was added and the tube taken off the magnetic plate for 3 minutes to ensure the complete re-suspension of beads. The tube was then placed back on the magnetic plate and purified DNA transferred into a 96 well optical plate (Applied Biosystems, USA) to be loaded onto the 3130xl Genetic Analyzer (Applied Biosystems, USA) to generate raw sequence data. At the end of the run, the raw sequence data was retrieved and further analyzed.

3.17 SEQUENCE DATA ANALYSES
Nucleotide sequences were edited and phylogenetic relationships between strains were established by comparing the sequences determined and aligning them using alignment programs Align IR V2 and CLUSTAL W. The degree of nucleotide sequence identity and of protein similarity between strains was determined using the default scoring matrices. Phylogenetic relationships between sequences were inferred by the maximum likelihood method with DNADIST/NEIGHBOUR of PHYLIP. The robustness of phylogenies was estimated by bootstrap analyses with 1,000
pseudoreplicate data sets generated with the SEQBOOT program of PHYLIP. Phylogenetic trees were constructed using neighbour-joining of PHYLIP and drawn using Neighbour-Joining Plot software. The MEGA 7 software package was used to perform most of these analyses.

3.18 DATA ANALYSIS

To obtain the risk factors from patients, the statistical analysis software version (SPSS 22.0) and stata version 13 (StataCorp LP, College Station, TX, USA) were used. Statistical significance was determined by using the chi-square test with p=0.05. Binary logistic regression model were used to obtain adjusted odds ratio for risk factors associated with RSV and HMPV positivity.

3.19 ETHICAL CONSIDERATIONS

The study ethical approval was obtained from the Ethical and Protocol Review Committee of the College of Health sciences, University of Ghana, (Appendix III). Informed assent were obtained from parents/guardians of study participants before they were enrolled. Written informed consent forms were obtained from parents or guardians of participating children. This was done by either thumb printing or signing of signature.
CHAPTER FOUR

4.0 RESULTS

4.1 PARTICIPANTS’ DEMOGRAPHIC

A total of 176 samples were collected from children below 5 years of age between September 2015 and November 2016.

There were 93 males study subjects (mean age 13.4 months, median 10 months, range 1-60 months) and 83 females (mean age 10.6; median 7 months and range 1-41 months). In terms of severity of disease, all patients were hospitalized with 12 cases obtained at the emergency departments (Table 6).

4.2 DETECTED VIRUSES AMONG STUDY PARTICIPANTS

The study detected 23/176 (13%) children were infected with one of two respiratory viruses (Table 6). RSV (20/176: 11.4%) and HMPV (3/176: 1.7%). RSV- A positive children (8/176: 4.5%) had a mean age of 11.6 months, median: 5 months, SD: 11.6 whereas RSV B positive children (12/176: 6.8%) had a mean age of 7.8 months, median: 2.5 months, SD: 11.7. Children positive for HMPV had a mean age of 14 months with median and SD of 12 months and 9.2 respectively. Statistically, there was no observed significant difference stratifying the groups by gender (p=0.106). No association (p=0.716) was found for children attending a child-day care center and those who did not (Table 6).
Table 6: Demographic characteristics of participants with respect to age groups, gender, educational status and patient category

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>No. of participants</th>
<th>RSVA Positives</th>
<th>RSVB Positives</th>
<th>HMPV Positives</th>
<th>Total Positives</th>
<th>P-value¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall Total</td>
<td>176 (100)</td>
<td>8 (5)</td>
<td>12 (7)</td>
<td>3 (1.7)</td>
<td>22 (13)</td>
<td></td>
</tr>
<tr>
<td>Age groups</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-6 months</td>
<td>76 (43)</td>
<td>4 (5)</td>
<td>9 (12)</td>
<td>1 (1)</td>
<td>13 (17)</td>
<td></td>
</tr>
<tr>
<td>7-11 months</td>
<td>26 (15)</td>
<td>1 (4)</td>
<td>1 (4)</td>
<td>0 (0)</td>
<td>2 (8)</td>
<td></td>
</tr>
<tr>
<td>11-23 months</td>
<td>47 (27)</td>
<td>1 (2)</td>
<td>1 (2)</td>
<td>1 (2)</td>
<td>3 (6)</td>
<td>0.41</td>
</tr>
<tr>
<td>24-48 months</td>
<td>26 (14)</td>
<td>2 (8)</td>
<td>1 (4)</td>
<td>1 (4)</td>
<td>4 (16)</td>
<td></td>
</tr>
<tr>
<td>49-60 months</td>
<td>1 (1)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>93 (53)</td>
<td>2 (2)</td>
<td>6 (7)</td>
<td>0 (0)</td>
<td>8 (9)</td>
<td>0.106</td>
</tr>
<tr>
<td>Female</td>
<td>83 (47)</td>
<td>6 (7)</td>
<td>6 (7)</td>
<td>3 (4)</td>
<td>14 (17)</td>
<td></td>
</tr>
<tr>
<td>Childs Educational status</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No school</td>
<td>152 (86)</td>
<td>7 (5)</td>
<td>12 (8)</td>
<td>2 (1)</td>
<td>20 (13)</td>
<td></td>
</tr>
<tr>
<td>Crèche</td>
<td>15 (8)</td>
<td>1 (7)</td>
<td>0 (0)</td>
<td>1 (7)</td>
<td>2 (14)</td>
<td>0.716</td>
</tr>
<tr>
<td>Nursery</td>
<td>8 (5)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>Primary</td>
<td>1 (1)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>Patients category</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hospitalized (cold cases)²</td>
<td>156 (93)</td>
<td>8 (5)</td>
<td>12 (8)</td>
<td>3 (2)</td>
<td>22 (14)</td>
<td>0.163</td>
</tr>
<tr>
<td>Not hospitalized (emergency)</td>
<td>12 (7)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td></td>
</tr>
</tbody>
</table>

¹ P-values were calculated using chi square method.

² Cases from the ward that are been managed specifically for ALRTI.
Of the total number of 176, 92 (52%) were diagnosed as having bronchopneumonia, 53 (30%) with acute respiratory tract infection followed by pneumonia and bronchiolitis, 20 (11%) and 11 (6%) respectively (Table 7). ARI is unclassified, for which it was not stated either Pneumonia or Bronchiolitis or Bronchopneumonia or multiple diagnosis. The proportions of patients with various clinical presentations diagnosed with the three viruses as shown in Table 7.

Table 7: Association of clinical diagnosis with RSVA, RSVB and HMPV infections

<table>
<thead>
<tr>
<th>Clinical Diagnosis</th>
<th>No. of Participants</th>
<th>RSVA n (%)</th>
<th>RSVB n (%)</th>
<th>HMPV n (%)</th>
<th>Total Positives n (%)</th>
<th>*P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pneumonia</td>
<td>20 (11)</td>
<td>2 (10)</td>
<td>3 (15)</td>
<td>1 (5)</td>
<td>6 (30)</td>
<td></td>
</tr>
<tr>
<td>Bronchopneumonia</td>
<td>92 (52)</td>
<td>4 (4)</td>
<td>5 (5)</td>
<td>0 (0)</td>
<td>9 (10)</td>
<td>0.04</td>
</tr>
<tr>
<td>Bronchiolitis</td>
<td>11 (6)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>Acute Respiratory Infection(ARI)</td>
<td>53 (30)</td>
<td>2 (4)</td>
<td>4 (8)</td>
<td>2 (4)</td>
<td>8 (15)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>176 (100)</td>
<td>8 (5)</td>
<td>12 (7)</td>
<td>3 (2)</td>
<td>23 (13)</td>
<td></td>
</tr>
</tbody>
</table>

* p-value analysis using chi square analysis
There was no association between education and occupational Status (p=0.184). The majority of parents or guardians was into non-formal sector and had only basic education. Table 8 shows the distribution of the educational level and occupation of study participants' parents and Guardians.

Table 8: Demographic characteristics of parents’ or guardians with child’s results

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>No. of participants</th>
<th>RSVA Positives n (%)</th>
<th>RSVB positives n (%)</th>
<th>HMPV Positives n (%)</th>
<th>Total Positives n (%)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall Total</td>
<td>176 (100)</td>
<td>8 (5)</td>
<td>12 (7)</td>
<td>3 (2)</td>
<td>22 (13)</td>
<td>0.71</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Parent or guardian's Educational level</th>
<th>No. of participants</th>
<th>RSVA Positives n (%)</th>
<th>RSVB positives n (%)</th>
<th>HMPV Positives n (%)</th>
<th>Total Positives n (%)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Un-educated</td>
<td>32</td>
<td>3 (9)</td>
<td>2 (6)</td>
<td>2 (6)</td>
<td>7 (22)</td>
<td>0.184</td>
</tr>
<tr>
<td>Primary</td>
<td>26</td>
<td>1 (4)</td>
<td>1 (4)</td>
<td>1 (4)</td>
<td>3 (12)</td>
<td></td>
</tr>
<tr>
<td>JHS</td>
<td>77</td>
<td>4 (5)</td>
<td>6 (8)</td>
<td>0 (0)</td>
<td>9 (12)</td>
<td></td>
</tr>
<tr>
<td>Secondary</td>
<td>23</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>Tertiary</td>
<td>18</td>
<td>0 (0)</td>
<td>3 (17)</td>
<td>0 (0)</td>
<td>3 (17)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Parent's or guardian work status</th>
<th>No. of participants</th>
<th>RSVA Positives n (%)</th>
<th>RSVB positives n (%)</th>
<th>HMPV Positives n (%)</th>
<th>Total Positives n (%)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-Formal</td>
<td>152</td>
<td>7 (5)</td>
<td>11 (7)</td>
<td>3 (2)</td>
<td>20 (13)</td>
<td>0.507</td>
</tr>
<tr>
<td>Formal</td>
<td>24</td>
<td>1 (4)</td>
<td>1 (4)</td>
<td>0 (0)</td>
<td>2 (8)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Parent's or guardian work</th>
<th>No. of participants</th>
<th>RSVA Positives n (%)</th>
<th>RSVB positives n (%)</th>
<th>HMPV Positives n (%)</th>
<th>Total Positives n (%)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Civil worker*</td>
<td>11 (6)</td>
<td>1 (9)</td>
<td>1 (9)</td>
<td>0 (0)</td>
<td>2 (18)</td>
<td>0.575</td>
</tr>
<tr>
<td>Health Worker*</td>
<td>2 (1)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0.592</td>
</tr>
<tr>
<td>Self employed*</td>
<td>133 (75)</td>
<td>6 (5)</td>
<td>9 (7)</td>
<td>3 (2)</td>
<td>17 (14)</td>
<td>0.942</td>
</tr>
<tr>
<td>Unemployed</td>
<td>30 (17)</td>
<td>1 (3)</td>
<td>2 (7)</td>
<td>0 (0)</td>
<td>3 (10)</td>
<td>0.671</td>
</tr>
</tbody>
</table>

---

3 Civil workers = government workers such as teachers, police, procurement officer.
4 Pharmacist
5 Self-employed= hairdressers, head porters, seamstress fishmongers, traders, beauticians, and bakers.
A total of 114 (65%) participants presented with fever out of which 7 (6%) were positive for RSVA, 7 (6) RSVB and 2 (2%) HMPV (Fig 5). The highest clinical symptom observed was cough among 148 (85%) of the study participants. Out of this, 6 (4%) had RSVA, 12 (8%) had RSVB, 3 (2%) had HMPV. Nasal conjuction followed closely among 134 (76%) of participants. Of these, 8 (6%) were positive for RSVA, 9 (7%) for RSVB and 3 (2%) for HMPV. Conjunctivitis and chest pain were seen in 2 (1%) and 4 (2%) participants and of these, 1 (50%) and 2 (50%) were positive for RSVB respectively. RSVB were found to be associated with all the clinical symptoms presented by the participants with the exception of participants with sputum production. Association of the 10 clinical symptoms and the presence of the various viruses increased from 5 (50%) being associated with HMPV to 9 (90%) of the clinical symptoms being associated with RSVB.
Figure 5: Frequency of clinical symptoms of children with ALRTI
A greater proportion of the positive ALRTI cases with RSV and HMPV infection were admitted at the hospital for longer than a week, although it was statistically insignificant (p=0.344) between the two RSV groups. Details of the types of infections and length of stay in hospital are shown below (Table 9).

<table>
<thead>
<tr>
<th>Days spent at the hospital</th>
<th>No. of Participants N (%)</th>
<th>RSVA n (%)</th>
<th>RSVB n (%)</th>
<th>HMPV n (%)</th>
<th>Total Positives n (%)</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 - 3 days</td>
<td>27 (15)</td>
<td>1 (4)</td>
<td>3 (4)</td>
<td>0 (0)</td>
<td>6 (22)</td>
<td>0.130</td>
</tr>
<tr>
<td>4 - 6 days</td>
<td>47 (27)</td>
<td>2 (4)</td>
<td>2 (4)</td>
<td>0 (0)</td>
<td>4 (9)</td>
<td>0.401</td>
</tr>
<tr>
<td>7 - 9 days</td>
<td>79 (44)</td>
<td>5 (6)</td>
<td>5 (19)</td>
<td>3 (4)</td>
<td>10 (13)</td>
<td>0.981</td>
</tr>
<tr>
<td>≥ 10 days</td>
<td>23 (13)</td>
<td>0 (0)</td>
<td>2 (9)</td>
<td>0 (0)</td>
<td>2 (9)</td>
<td>0.575</td>
</tr>
<tr>
<td>Total</td>
<td>176 (100)</td>
<td>8 (5)</td>
<td>12 (7)</td>
<td>3 (2)</td>
<td>22 (13)</td>
<td></td>
</tr>
</tbody>
</table>

The chi test was used to determine dependents on length of stay.
Feeding characteristics of participants before and after 6 months was not statistically significantly associated with the type of food and viral infection (Table 10)

Table 10: The association of RSV and HMPV infection on breastfeeding

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>No. of participants</th>
<th>RSV Positives n (%)</th>
<th>HMPV Positives n (%)</th>
<th>Total Positives n (%)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall total</td>
<td>176(100.0)</td>
<td>20(100.0)</td>
<td>3(0.0)</td>
<td>23(100)</td>
<td></td>
</tr>
<tr>
<td>Feeding before 6 months</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exclusive breast feeding</td>
<td>168(95.5)</td>
<td>19(95.0)</td>
<td>3(100.0)</td>
<td>22(97.5)</td>
<td>0.741</td>
</tr>
<tr>
<td>Formula foods</td>
<td>4(2.3)</td>
<td>1(4.5)</td>
<td>0(0.0)</td>
<td>1(2.5)</td>
<td></td>
</tr>
<tr>
<td>Lactogen</td>
<td>1(0.6)</td>
<td>0(0.0)</td>
<td>0(0.0)</td>
<td>0(0.0)</td>
<td>0.741</td>
</tr>
<tr>
<td>Koko</td>
<td>3(1.7)</td>
<td>0(0.0)</td>
<td>0(0.0)</td>
<td>0(0.0)</td>
<td></td>
</tr>
</tbody>
</table>

6 Koko = porridge prepared with millet, corn dough,
Monthly distribution of sample collection from September 2015 through to November 2016. RSV was characterized by two peaks in the month of October and December (Figure 6). The blue colour indicates the number of participants, red indicates RSV A positives, Green represents RSV B patients and HMPV is indicated in yellow colour.

**Figure 6:** Monthly distribution of RSVA, RSVB and HMPV over the study period
The 176 participants presenting with ALRTI, RSVA, RSVB and HMPV were found to be 8 (4.5%), 12 (6.8%) RSVB and 3 (1.7%) HMPV respectively as shown in Figure 7.

![Prevalence of RSVA, RSVB and HMPV](image)

**Figure 7:** Prevalence of RSVA, RSVB and HMPV in children with ALRI

Binary logistic regression was used to calculate the odds ratio for risk factors. There was no significant difference between individuals infected with any of the viruses and gender. It was observed that individuals within the lower age category (0 to 12 months) were 6 times more likely to be infected with RSV-B compared to individuals in the higher age category (13 to 60 months) even though this test was not significant (p = 0.071) (Table 11).
### Table 11: Risk factor associations with RSVA, RSVB and HMPV infections among children under five years

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>RSVA OR (95% CI)</th>
<th>p-Value</th>
<th>RSVB OR (95% CI)</th>
<th>p-Value</th>
<th>HMPV OR (95% CI)</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gender</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>0.526 (0.122-2.271)</td>
<td>0.389</td>
<td>0.895 (0.277-2.892)</td>
<td>0.853</td>
<td>0.445 (0.039-5.000)</td>
<td>0.512</td>
</tr>
<tr>
<td>Female</td>
<td>-</td>
<td>Ref</td>
<td>-</td>
<td>Ref</td>
<td>-</td>
<td>Ref</td>
</tr>
<tr>
<td><strong>Age category</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 to 12 months</td>
<td>0.935 (0.216-4.048)</td>
<td>0.928</td>
<td>6.752 (0.851-53.587)</td>
<td>0.071</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>13 to 60 months</td>
<td>0.566 (0.289-1.109)</td>
<td>0.097</td>
<td>1.003 (0.595-1.688)</td>
<td>0.992</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Parent education</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>feeding before 6 months</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>feeding after 6 months</td>
<td>0.987 (0.424-2.297)</td>
<td>0.976</td>
<td>0.252 (0.083-0.764)</td>
<td>0.015</td>
<td>0.41 (0.101-1.659)</td>
<td>0.211</td>
</tr>
<tr>
<td>Environmental smoking</td>
<td>1.448 (0.167-12.57)</td>
<td>0.543</td>
<td>0</td>
<td>0</td>
<td>0.998 (0.96-1.003)</td>
<td>0.579</td>
</tr>
<tr>
<td>Parental smoking</td>
<td>1.05 (1.015-1.087)</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0.999 (1.018)</td>
<td>0.702</td>
</tr>
<tr>
<td>Siblings with RTI</td>
<td>1.282 (0.151-10.857)</td>
<td>0.819</td>
<td>0.218 (0.064-0.749)</td>
<td>0.009</td>
<td>1.021 (0.997-1.045)</td>
<td>0.456</td>
</tr>
<tr>
<td>windows present in room</td>
<td>1.015 (1.015-1.087)</td>
<td>0.582</td>
<td>0.321 (0.034-3.011)</td>
<td>0.295</td>
<td>1.018 (0.998-1.04)</td>
<td>0.74</td>
</tr>
</tbody>
</table>
4.3 MOLECULAR CHARACTERIZATION

4.3.1 Gel electrophoresis

Of the 176 samples run, 8 were positive for RSVA, 12 for RSVB and 3 for HMPV. The remaining samples showed no amplification bands and therefore were negative for any of the tested viruses. Sample gel photographs for RSV A, RSV B and HMPV are shown in Figures 8, 9 and 10 respectively.

**Figure 8:** An electrophoregram (1.5% agarose gel in 1X TAE buffer) of RSV A amplification. Lane 1 contained the 100bp DNA ladder (Biolabs, New England). Lanes 2 is a positive control; Lanes 3 is a negative control and lanes 4 to 6 contained positive samples from study participants. Expected size of positive amplification product is 593bp.
Figure 9: An electrophoregram of amplified RSV B.

Agarose gel electrophoresis (1.5%) was run using 1X TAE buffer. Lane 1 contained the 100bp DNA ladder (Biolabs, New England). Lanes 2 to 4, contained samples from study participants. Lane 5 has a negative control whilst lane 6 contained a positive control. The expected size of the positive products of RSV B was 509bp. There was no amplification of Lane 2 containing a sample of a study participant.
**Figure 10: Gel electrophoresis of HMPV**

An Agarose gel (1.5%) was run using 1X TAE buffer. Lane 1 contained the 100bp DNA ladder (Biolabs, New England). Lanes 2 and 3 contained a positive control and negative controls respectively. Lanes 4 to 6 contained samples from study participants. The expected size of the positive product was 527bp for second round PCR products.
4.3.2 Phylogenetic analysis
The second hypervariable region (VR2) of the G protein gene was sequenced for 5 RSV group A and 7 RSV group B viruses. The sequences were aligned in MEGA 7 and compared with reference sequences representing the different genotypes. RSV group A viruses belonged to the "novel" genotype ON1, whilst RSV group B viruses belonged to genotype BA9 (Figure 11).
Figure 11: Neighbour joining trees representing phylogenetic analysis of RSV genotypes isolated in Ghana between 2015 and 2016. This tree was constructed using the Neighbor-Joining method (Saitou N. and Nei 1987). “The optimal tree with the sum of branch length = 0.90679067 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) and are in the units of the number of base substitutions per site. Sequences from this study are shown in bold green color and designated by the geographic location (GHA-PML), 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.
The nucleotide difference between the RSV-A study samples and ON1 genotype is shown in figure 12. Seven nucleotide changes were observed. Two of these nucleotide changes in the G-gene (at position 66 G→A, and at position 84 T→C) were synonymous (silent) and did not result in any change of amino acids. The other five nucleotide changes were non-synonymous (amino acid altering) at positions 95 T→G, 151 G→A, 158 C→T, 260 T→C, 310 T→A. These nucleotide mutations resulted in amino acid substitutions at amino acid positions 32, 51, 53, 87, and 104, where isoleucine (I) replaces serine (S), glutamic acid (E) replaces lysine (K), threonine (T) replaces isoleucine (I), leucine (L) replaces proline (P), and serine (S) replaces threonine (T), respectively. The amino acid change at position 53 occurred in two samples only (PML-GHA-16-141, PML-GHA-16-147) while the remaining four amino acid substitutions occurred in all samples.

**Figure 12:** Graphic view of nucleotide changes between RSV-A study samples and RSV-A ON1 reference genotypes within the G gene.
There were 20 nucleotide changes found in RSV-B as compared to the reference genotype BA IX. Of these, 3 occurred in all five samples at positions 12 where T was substituted for C, 292 where T replace C and at position 301 where A was replaced by C. One nucleotide change was also found in four samples at position 81, and 2 in three samples at positions 11 and 301. As seen in figure 13, non-synonymous amino acid changes were found at 12 positions. These include a change at amino acid position 4 which transformed proline to leucine in samples PML-GHA-15-12, PML-GHA-15-22 and PML-GHA-15-33, threonine to isoleucine at position 42 in three samples and tyrosine to histidine at position 75 in two samples (Figure 13).

**Figure 13:** Amino acid changes between RSV-A study samples and RSV-A ON1 reference genotype
Nucleotide difference between the RSV-B study samples and BAIX genotype. Several nucleotide changes were observed with 3 occurring in all samples at positions T12C, C297T, A305G and another 4 occurring in more than half or 50% of the samples at positions C11T, C81T, C125T and T301C. Of these nucleotide changes, 3 (C11T and T12C, C125T) were non-synonymous (amino acid altering) which resulted in amino acid changes P4L and D42I respectively (Figure 14). The others C81T, C297T and T301C were synonymous (silent) (Figure 14).

**Figure 14.** Graphic view of nucleotide changes between RSV-B study samples and RSV-B BAIX reference genotypes
Figure 15: Amino acid changes between RSV-B study samples and RSV-B IX reference genotype. Dots represent positions with similar amino acids in study sequences as the reference sequence. Alphabets are amino acid names and show positions where the amino acids in the study sequences are different from that at the same position of the reference sequence.
CHAPTER FIVE

5.0 DISCUSSION AND CONCLUSIONS

Acute lower respiratory tract infections (ALTRIs) still remain a significant public health problem worldwide (Lu et al., 2013). Several studies have shown that RSV is a frequent cause of acute respiratory infections in children under 5 years, contributing 21 to 68% of the cases investigated. However, in this current study the prevalence of RSV was relatively low (11.4%). Similarly in another study report, the occurrence of RSV was low (8.1%) among patients hospitalized with ALRTIs (Naorat et al., 2013). Even so, over 70% of the RSV cases in this study report involved patients less than five years old. One reason that may contribute to variations in RSV prevalence is the differences in the methods used to establish diagnosis. For example a study by You and his colleagues (You et al., 2017) to optimize diagnosis and efficiency of respiratory tract infections, designed a one step Triplex Real time RT-PCR to detect HMPV and RSV from 222 RTI participants. Their results demonstrated high detection of RSV in 68 (30.1%) and 18(8.1%) in HMPV patients. Another study by Adiku and others (2015), which used a conventional multiplex RT-PCR method obtained a prevalence rate of 18% for RSV. From this study, the detection of the RSV was made possible using a convectional nested singleplex RT-PCR assay. Therefore the 11.4% positivity rate of RSV observed confirms the prevalence variations that comes with use of different methods. Besides other factors such as study site, period of year, duration of study, seasonal variation and number of samples, health care setting may play a role. As compared to RSV, HMPV infection rate from this study was low with a prevalence of 1.7%. Several reports have generally
reported low rates for HMPV. Despite their low prevalence HMPV may also cause severe infections in ALRTI patients (Arnott et al., 2011; Chow et al., 2016; Mullins et al., 2004).

Both males and females were equally infected with RSV A and B indicating an equal chance of each gender acquiring the infection. A recent study from Senegal also revealed that there were no striking differences in RSV detection rates between male and female patients (Fall et al., 2016). On the contrary to these finding, some previous studies from Ghana and the Gambia reported a male predominance for RSV infection in children, especially in the younger age group less than one year old. It is however not clear why this is so in nature (Forgie et al., 1992; Obodai et al., 2014). This study also reports the predominance of RSV B infection in children hospitalized other than RSV A. Due to the high number of RSV B infection comparison to RSV A with only 8 children the epidemiological data is therefore not conclusive. RSV B children were found to have a mean age lower than that of A (11.06; p<0.05), a finding similar to that of Lamarão et al., 2012. He also reported a predominance of RSV B infection in younger children, but the reasons are still unknown. Nevertheless, there was no statistically significance difference between these two subgroups, although RSV A infections occurred more frequently in females than males there is no explanation for this variation.

The “Novel” ONI strain, a new emerging strain is spreading partly due to global associated travel respiratory illness. It has also been reported in countries such as Kenya (Otieno et al., 2017), Germany (Schnitzler, 2014), Italy (Pierangeli et al., 2014) and South Africa (Pretorius et al., 2013), and like the BA prototype (Parveen et al., 2006) hence replacing all old subtypes as reported to be seen in countries such as Kenya,
Canada, Brazil and India (Parveen et al., 2006). This genetic variation of RSV strains also contributes more to the ability to infect people yearly (Reiche & Schweiger, 2009).

RSV group dominance is consistent shifts has been reported in various studies. From a previous study in Ghana, RSV group A and B viruses alternately predominated in two consecutive years or seasons, RSVB in 2013 and HRSVA in 2014 (Obodai, 2016, Unpublished data). Similarly, this current study has observed that RSV group B viruses predominated in 2015, and alternately in 2016 group A viruses prevailed. Hence there appears to be a regular yearly-cyclic-pattern of RSV group dominance. Nevertheless, long term studies over a couple of years will confirm the HRSV circulation patterns in Ghana. In Belgium 15 consecutive years of RSV surveillance (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 (Houspie et al., 2013). Molecular characterization showed that all RSV A belonged to genotype ON1 with a prevalence 4.5% and RSV B belong to genotype BA9 with a prevalence of 6.8%. The BA 9 are the predominant RSV B genotypes circulating worldwide and has also been reported to have circulated in Ghana in 2006 (Obodai et al., 2014). Globally, ON1 prevalence seems to vary by location. In Canada, where it was first reported in 2010, the prevalence of ON1 has remained stable at 11%–13% (Duvvuri, Granados, Rosenfeld, Bahl, & Eshaghi, 2015). Similarly, other countries have also reported ON1 prevalence rates less than 20%. In South Africa (Pretorius et al., 2013) and United states (Avadhanula et al., 2015). The varying prevalence suggests that although ON1 is spreading worldwide and ecological differences and host factors may determine the spread.
RSV accumulates amino acid changes over time (Cane & Pringle, 1995), and the observed amino acid changes in the ON1 viruses of RSV A and BA 9 of RSV B in this current study are suggestive of similar accumulation. A reason for the long-lasting circulation of this genotype might be due to mutations in their amino acid sequences. For all the genotypes, the amino acid divergence was greater than the nucleotide divergence since more non synonymous mutations were observed indicating a positive selection pressure for those viruses. Contrary to this, selection pressure for ON1 seem due to the existence of more synonymous mutations. The significance of this observation is not known so far. Three nucleotide positions 32, 51 and 87 within the G-protein region analyzed were found to have nucleotide substitutions that resulted in amino acid changed from isoleucine to serine, glutamic acid to lycine and leucine to serine respectively, but their functions are not clear. However other reports have shown that such an amino acid mutation at certain position such as Glu-232-Val could result in RSV-A escape mutants and in loss of reactivity to a specific monoclonal antibody (Cane & Pringle, 1995). Likewise for RSV B, nucleotide substitutions that resulted in amino acid changed for position 4, 42, 75 which transformed proline to leucine, threonine to isoleucine and tyrosine to histidine respectively.

Generally it is known that in lower respiratory tract infections, risk factors such as young age between the ages of 0–12 months, male gender, passive smoking, sharing a bedroom with other children aged 0–5 year with respiratory tract infection, children attending child-day care centers are strongly associated with both upper and lower respiratory tract infections. The present study assessed these risk factors that predisposes children below five years to ALRTI. By using univariate and multivariate analysis there were no
significance differences between these variables detected. Nevertheless, these analysis were based on a relatively small number of patients suggesting that the outcome observed may not be a true reflection, furthermore, majority of infants aged between 0-6 months (43%) were the most affected, and the rate of infection decreased with increased age. This suggest that age may be a contributing or risk factor of ALRI and are in line with reports of several other studies which also showed the similar pattern with the greater proportion of the children being less than 12 months old (Koch et al., 2003; Macedo et al., 2003 Lamarão et al., 2012; Okiro, Ngama, Bett, & Nokes, 2012; Moyes et al., 2013; Mc cracken et al., 2014). For infants <12months with severe disease, it tends out that there were more likelihoods of having RTI infection which may be due to their immature immune system, hence the probability of having more children being infected in this age group. However, HMPV infected individuals were more advanced in age than infected RSV individuals and this findings is in consonance with work done elsewhere which appeared to have high frequency of HMPV infected children between 6 to 24 months old (Akhras et al, 2010; Cuevas et al., 2009).

Breast milk plays a role in the development of a neonate and has been proven to be a crucial factor for mammalian survival whilst maternal antibodies are also transferred to the neonates during feeding (Walters & Ward, 2004). However from this study, there was no association between ALRTI and breastfed or non-breastfed children even though about 95% of study participants were exclusively breastfed.

The incidence of 1.7% of positive HMPV specimens among those tested was slightly lower than the 3.9% reported in one study in New York, USA (Mullins et al., 2004).
Although other studies have found that HMPV incidence may vary from year to year. The duration of this (< 1 year of sample collection) did not allow us to evaluate this.

There was no co-infection between RSV and HMPV. This is in sharp contrast to previous studies that reported co-infections of RSV and HMPV (Semple et al., 2005; Al-sonboli et al., 2006; Kim et al., 2010; Ramirez et al., 2014b; Moattari, Aleyasin, Emami, Fyruzi, & Pirbonyeh, 2015).

The number of admissions between July and October is related to the environmental factors associated with cold temperatures, especially domestic confinement (Macedo et al., 2003). These conditions are particularly important for the seasonality of viral infections; particularly RSV as observed in the present study. The study period experienced some rainfalls from the month of October to November 2016 and it was in October that recorded the highest detection of most viral infections with the commonest being RSV. This phenomenon has also been reported in other developing countries (Kwofie et al., 2012; Moattari et al., 2015).

Bronchopneumonia, pneumonia and acute respiratory infection were the most reported complications associated with ALRI infections in the present study, which concurs with previous studies, (Adiku et al., 2015). Some studies have also named bronchiolitis, a predominantly common viral disease of RSV being the leading pathogen of this condition (Azkur et al., 2014). From this present study, there was an association between clinical diagnosis and ALRI infection. This study suggests and agrees with other findings, although there were some differences in terms of numbers in this study. Furthermore, seropositivity for HMPV is said to approach by 100% by the age of 5 to 10
years of age and this explains the low turnout of positive results for HMPV in this study. Nevertheless, prevalence rates in hospitalized children can range from 1.5% to 17%. (Owor et al., 2016).
5.1 LIMITATIONS

- The inability to sequence HMPV from all the 3 patients sampled was a drawback since this would have provided more data as to which genotype circulated during the study period and among the study population.
- The study was limited to only one sampling site hence this study would not reflect the true HMPV and RSV situations in the whole country.
5.2 CONCLUSION

The study concludes that:

- Respiratory Syncitial Virus and Human Metapneumo Virus contribute considerably to ALRI among Ghanaian children below five years old.

- Bronchopneumonia and ARI are the most common clinical manifestations among patients that tested positive for RSV and HMPV.

- Having siblings with ALRIs may increase the risk of a child having RSV infection.

- Sequencing of RSV isolates to determine their genetic diversity was performed for the first time in Ghana. RSV group A and B viruses were found to be co-circulating. RSV-B belonged to genotype BA9, whilst the most predominant genotype found to be circulating for RSV A viruses was the “novel” genotype ON1.

- Severity of the disease could not be correlated to the duration of stay in hospital.
5.3 RECOMMENDATIONS

Based on the findings from this study, the following are recommended:

- Further research work on RSV and HMPV needs to be done across the country to determine the role of both viruses and extended for about 5 years in order to establish the seasonal pattern of these viruses in Ghana as well provide baseline data for evaluation of RSV vaccines.
- There is need to establish diagnostic systems across health facilities to detect both viruses in ALRI cases towards effective clinical management.
- There should be sequencing of RSV and HMPV genes in order to know their diversity of the viral genome in circulation. This would be beneficial for the development of vaccines.
- Public health messages should be re-packaged in a way to pre-inform the public on what to do during the peak seasons of these viruses.
- The role of HMPV in older children could be further investigated to broaden our knowledge on its association with ALRI.
REFERENCES


http://doi.org/10.1093/infdis/jis333

http://doi.org/10.1128/JCM.00187-06


http://doi.org/10.1093/infdis/jit477


APPENDICES

APPENDIX 1

PARENT/LEGAL GUARDIAN INFORMED CONSENT FORM

INFORMED CONSENT FORM FOR PARENTS OR GUARDIANS OF CHILDREN BELOW YEARS WHO HAVE ACUTE LOWER RESPIRATORY TRACT INFECTION

Name of organization: School of biomedical and allied Health Sciences (SBAHS), Korle-Bu, Accra.

Project Title: Detection and Characterization of human metapneumovirus and Respiratory Syncytial VIRUS (RSV) Infection in Ghanaian Children below five years with Acute Lower Respiratory Tract infection

Principal Investigator: Ms Anna Aba Hayford

Address: Medical Microbiology Unit. School of Biomedical and Allied Health sciences. P.O.Box 43, Korle -Bu

GENERAL INFORMATION AND INFORMED CONSENT

I am seeking your consent to enrol your ward/child in a study in which nasal swabs specimen shall be collected with a sterile tube to investigate in Respiratory Infections in children. This is a research which is being conducted at the Medical Microbiology Department of the School of Biomedical and Allied Health Sciences and the research aims at detection and characterizing of Human Metapneumovirus virus and Respiratory Syncytial Virus (RSV) a pathogen that causes respiratory infection particularly in children below 5 years. After accepting to participate in the study, you have the option of changing your mind and withdraw from the study at any a time without any objection. This will not prevent you from being attended to at the health facility. In the course of the
study, you may be made to answer questions on behalf of your child. I may also review your medical records.

**Discomfort and Risk**

There is mild discomfort in obtaining samples from your child. Maximum care will be taken by a qualified personnel in collecting the samples. You may however feel the discomfort of being asked to answer some questions.

**Patient Benefit**

The study would help to promote knowledge and improve patient management and also help identify risk factors associated with RSV and HMPV infections and better manage such cases in the Future.

**Confidentiality**

The information about the research will be held highly confidential. Numbers will be assigned to any information collected from your child and the identity will not be revealed. Findings of the study will be reported but not with your child’s name assigned to it and other personal details. Your decision to allow your child to partake in the research is entirely voluntary and you can withhold your consent without fear. This will in no way prevent your child from receiving treatment.

**Future Use of Biological Specimen**

We would like to keep/store leftover of your child's specimen for future studies on other Respiratory diseases if you agree. Your child's name will not be recorded on the specimen. However, your child can still participate in this study even if you do not want us to store his/her specimen.

**CONTACTS**

This proposal has been reviewed and approved by the Ethical and Protocol Review Committee of the college of Health Sciences, which ensures that the participants of the
study are protected from harm. If you have any concerns about your child’s participation you can contact Anna Aba Hayford on 0244-767162

PARENT/LEGAL GUARDIAN'S AGREEMENT

My child has been invited to participate in this research and I have fully been informed of the above-described study with its possible benefits and risk. I have been made to understand that, the study is entirely voluntary and that I can decide to participate or withdraw from it at any time without any consequences to the subsequent treatment and care I will receive at the health facility. I consent voluntarily to my child’s participation in the research.

Volunteer Agreement

I agree that my child .................................................................should participate as a volunteer.

---------------------------------------------------------------
Date                            Name and signature/thumprint of parent/guardian

---------------------------------------------------------------
Date                            Signature of Witness
APPENDIX II

HMPV AND RSV SURVEY QUESTIONNAIRE FORM

Form Number :

Hospital Folder Number ……………………………………………………..

1. Name………………………………………………………………………………………………………………………………………

2. Age…………………..

3. sex:  M / F

4. date of visit: ……………………………………………………………………………………………………………………..

5. Date of Onset/Acute Onset of disease ……………………………………………………………..

6. Date of Sample Collection……………………………………………………………………………………………………

7. Address (place of residence) ………………………………………………………………………………………………………

8. Educational background of child:  a) None  b) Crèche   c)Nursery   d) Primary

9. Educational background of parent/guardian: (a)None   (b) Primary   (c) J.H.S
   (d) Secondary   (e) Tertiary

10. Parent /guardian occupation: (a) Formal............................. b) Non formal………………………………………………………………………………

11. Clinical Features of Patient

   Respiratory symptoms presented by child: (a) dyspnea  (b) Fever  (c) Fast breathing  (d) wheezing  (e) cough  (f) cough and Sputum production  (g) sore throat  (h) conjunctivitis  
   (i) otitis media  (j) Chest pain  (k) nasal discharge  (l) difficulty in breathing  (m) Difficulty in feeding

   12. Non respiratory symptoms presented by child: (a) Headache  (b) Confusion  (c) Myalgia  (d)Abdominal pain  (e) Nausea  (f) Vomiting  (g) Diarrhea

   (h) others…………………………………………………………………………………………………………………………………..

13. Has child been on any medication for treatment of illness, please specify: Y/N

…………………………………………………………………………………………………………………………………………………

14. Medical history, has your child suffered from any previous disease: Y/N

…………………………………………………………………………………………………………………………………………………
Please specify........................................................................................................................................

15. Was the child born a) Preterm  b) full term .................................................................

16. Feeding history up to 6 months: a) Exclusive breast feeding  b) others......................

17. What kinds of food did you feed your child after six months: a) breast milk  b) others.................................................................

18. Does child sleep alone: (a) Yes   ( b) No

19. If no, how many in a room:....................................................................................

20. Number of windows to a room.................................................................................

21. Is there any sibling with ALRTI: (a) Yes   (b) No

22. If yes, how many..................

23. Is there any form of environmental smoke exposure/ passive tobacco smoking of either parents:   a) Yes  b) No

24. Clinical diagnosis (to be documented from patient folder): a) Pneumonia b) Bronchopneumonia  c) Bronchiolitis d) Acute Respiratory Infection  e) Others........................................................................................................................................

25. Medical Exam Temp:  a)Temp(°C)...............  b)Respiratory rate........................................ (c) others....................................................................................................

26. Did your child/ward receive a ventilatory support? a) Yes  b) No

27. Date of Discharge--------------------------(this will be documented from the Admissions books of the Hospital)
APPENDIX III

Ethical Clearance Certificate from COLLEGE OF HEALTH SCIENCES Ethical protocol and review committee
APPENDIX IV

PERMISSION GRANTED TO USE PML AS A COLLECTION SITE BY THE REGIONAL GHANA HEALTH SERVICE DIRECTOR

THE MEDICAL DIRECTOR
RIDGE HOSPITAL
ACCRA

THE MEDICAL SUPERINTENDENT
PRINCESS MARIE LOUIS (PML) HOSPITAL
ACCRA

RE: LETTER OF INTRODUCTION – MS. ANNA ABA HAYFORD

The Regional Health Directorate has given approval to enable Ms Anna Aba Hayford, an MPhil student in the Department of Medical Microbiology, School of Biomedical and Allied Health Sciences, College of Health Sciences, University of Ghana to collect nasopharyngeal swabs and data on patients’ clinical history in your facility to enable her to undertake her project on the topic: “Detection and characterization of Human Metapneumovirus and Respiratory Syncytial Virus in Children Less Than Five Years Old With Acute Lower Respiratory Tract Infections” as per the attached.

Kindly accord her with the needed assistance to enable her to complete the exercise successfully.

Thank you.

DR. LINDA A. VANOTOO
REGIONAL DIRECTOR OF HEALTH SERVICES
GREATER ACCRA

cc: Ms. Anna Aba Hayford
School of Biomedical And Allied Health Sciences
College of Health Sciences
University of Ghana
Department of Medical Microbiology
APPENDIX V

Materials

A: Reagent for laboratory work

QIAamp viral RNA kit (QIAGEN, USA)

Absolute ethanol (molecular biology grade) [SIGMA, USA]

Nuclease-free water (Ambion, USA)

primers (Eurofilms, Germany)

Taq and DNTPs (invitrogen, Germany)

Agarose (SIGMA, USA)

Ethidium bromide (SIGMA, USA)

Tris-Acetate-EDTA (TAE) [Ambion, USA]

DNA molecular weight 100bp ladder (Biolabs, New England)

QIAquick PCR purification kit (QIAGEN, USA)

Big Dye Terminator Cycle Sequencing Kit vs. 3.1 (ABI, USA)

AgenCourt CleanSeq Dye Terminator Removal kit (Beckman Coulter, USA)

Sequencing Buffer with EDTA 5X (ABI, USA)

B. Laboratory equipment

Biological Safety Cabinet Class II (LABGARD, USA)

Biosafety Cabinet Class IIA (AirTech Services, India)

Vortex Genie-2 (Scientific Industries, USA)

Autoclave SS-325 (Tomy, Japan)
Microcentrifuge 5415D (Eppendorf, USA)

AirClean 600 PCR Workstation (AirClean Systems, USA)

GeneAmp PCR System 2700 and 2720 (ABI, USA)

Microwave oven (LG Electronics Inc., Ghana)

Gel logic 100 Imaging System (Eastman Kodak Company, USA)

Genetic Analyzer 3130 (ABI, USA)

C. Sequence analysis software

Clustal W (https://www.megasoftware.net/mega4/WebHelp/.../clustalw/hc_clustalw.htm)

MEGA 7.0 (www.megasoftware.net)