CHARACTERIZATION OF HOSPITAL ACQUIRED RESPIRATORY SYNCYTIAL VIRUS FROM SELECTED REFERRAL HOSPITALS IN KENYA (2009- 2011)

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Characterization of Hospital Acquired Respiratory Syncytial Virus from Selected Referral Hospitals in Kenya

(2009- 2011)

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other University.

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DEDICATION

This thesis is dedicated to the almighty God, for His grace and guidance and to my dear husband Amos and my children Noel and Olivia for their daily encouragement in improving my life and to my parents Mr. and Mrs Mayieka for instilling in me the value of prayer, hard work and persistence in life.

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ABBREVIATIONS AND ACRONYMS

ABI	-	Applied Biosystems
CDC	-	Centres for Diseases Control and Prevention
cDNA	-	Complementary Deoxyribonucleic Acid
Ct	-	Cycle Threshold
EKC	-	Epidemic Keratoconjuctivitis
HAI	-	Hospital Acquired Infection
HAdV	-	Human Adenovirus
rHAI	-	Respiratory Hospital Acquired Infection
HCW	-	Health Care Workers
hMPV	-	Human Metapneumovirus
IPC	-	Infection Prevention and Control
JKUAT	-	Jomo Kenyatta University of Agriculture and Technology
KNH	-	Kenyatta National Hospital
MOPHS	-	Ministry of Public Health and Sanitation
MDH	-	Mbagathi District Hospital
NNIS	-	National Nosocomial Infections Surveillance
NNPGH	-	New Nyanza Provincial General Hospital
PCR	-	Polymerase Chain Reaction
PIV	-	Parainfluenza Virus
RNA	-	Ribonucleic Acid
RSV	-	Respiratory Syncytial Virus
μl	-	Microlitre
WHO	-	World Health Organisation

ABSTRACT

Respiratory Syncytial Virus (RSV) is a major cause of respiratory illness in all ages but occurs more often in children under 2 year. This study evaluated the genetic diversity of G protein gene in samples collected from petients who developed influenza like illnesses while admitted at Kenyatta, New Nyanza and Mbagathi District Hospitals then tested positive for RSV. nasopharyngeal and oropharyngeal samples were collected from patients with new-onset fever ($\geq 38°C$) and either cough or sore throat, after being afebrile for at least three days in the wards. Specimen were tested for RSV alongside other viruses using real time polymerase chain reaction (RT-PCR).Those positive were further Subtyped as A or B using the same method. Where subgroup was identified, the attachment G-protein was sequenced and phylogenetically analyzed. Among 248 specimen tested, 37 (14.9%) were RSV positive, 17(45.9%) sequenced successfully. Out of the 17, 9(53%) were RSVA subtype, one being ON1 genotype and 8 NA1 genotype. The remaining 8(47%) were RSV B subtype all genotyped as BA9 and over 80% of the isolates were genetically related. Most positive specimen were from the male patients (59.5%) and from KNH (59.5%).Those aged 5years and above had 79% less odds of contracting RSV than those aged 2 years, Both RSV subtype A and B were observed to be circulating during the study period. The RSV A was circulating in two genotypes, ON1 and NA1, while all RSV B were of genotype BA9 with 69% of the isolates clustering by time and place. The latter findings suggest presence of nosocomial transmission of RSV in the hospitals. This study concludes that Infection control intervention should target those below 2years and recommends further research targeting healthcare workers and visitors who come to see patients in the hospitals.

CHAPTER ONE

INTRODUCTION

1.1 Background Information

Health-care associated infections (HAI) also called nosocomial infections occur in a patient in hospital or other healthcare facilities in whom the infection was neither present nor was incubating at the time of admission. Hospitalised individuals are often immunocompromised and are prone to infection due to invasive examinations and treatments which create potential route for transmission, and patient care practices. These factors contribute to the frequency of nosocomial infections. There are various types of infections. These include infections of the urinary tract, surgical site, the respiratory tract, blood, the skin, gastrointestinal tract and the central nervous system (CDC, 1996).

Among the various pathogens causing HAI, bacteria have been found to be the most common. A number of viruses have also been documented to cause HAI (Aitken & Jeffries, 2001). Nosocomial viral transmission can occur via respiration (influenza, Respiratory Syncytial Virus, human Metapneumovirus, Parainfluenza Virus , Measles, mumps and rubella) and the fecal oral route (rotavirus, enterovirus, hepatitis A and hepatitis E). Surveillance data reported by the National Nosocomial Infections Surveillance (NNIS) System for 1993-1998 shows a continuing increase in antimicrobial-resistant pathogens associated with nosocomial infections in ICU patients from U.S. hospitals (CDC NNIS system, 1999).

The occurrence of Health-care associated infections is a threat to the health and safety of both patients and health care workers (Kent S.,1996) . The infection rates are considered as an indicator of the quality of health care and appropriateness of infection control measures.Health-care associated infections have substantial impact on morbidity and mortality and represent an important public health threat (Yalcin, 2003), and increase both direct and indirect costs of patients' care (Jarvis, 1996). The increased length of stay for infected patients is the greatest contributor to cost (Pittet *et al*, 1994), they add to the imbalance between resource allocation for primary and secondary health care by diverting scarce funds to the management of potentially

preventable conditions; they necessitate additional laboratory procedures, additional use of drugs and the need to use the isolation room. . About 5% of the world population is hospitalized annually with about 10% of them dying of HAI. (Burke, 2003). It is estimated that between 5-10% of patients admitted in hospitals in the developed countries acquire HAI (WHO,2011). In the USA, 2 million HAI cases occur per year leading to an estimated US$ 4.5–5.7 billion in additional healthcare expenditure and about 90,000 deaths (Starfield, 2000) while England's 100,000 HAI cases per annum is estimated to cost the National Health Service a minimum of £1 billion per year (Mayor, 2000). These infections can also be transferred to the community through discharged patients, visitors or healthcare workers and may cause significant disease in the community.

Nosocomial infections are largely undocumented as a health risk in developing countries, but they are likely to become public health priorities as awareness of their occurrence increases (Aiken *et al.,* 2011). At any given time, the prevalence of health-care associated infections varies between 5.7% to 19.1% in low and middle income countries.Average prevalence is significantly higher in high than in low quality studies (15.5%-8.5% respectively).Unlike more affluent countries, infectious diseases continue to pose a heavy burden of morbidity and mortality in developing nations (Shears, 2007). Scant data suggests that in resource-poor settings, 10-30% of all admissions result in HAIs (Pittet & Donaldson, 2005). Although respiratory viral infections are much studied in developed countries and their impact on health care is well understood, there is a gap on information on the burden of respiratory viral infections as a contributor to HAI in developing countries (Rudan *et al*, 2005)

Nosocomial respiratory infection is the second most common cause of hospital-acquired infections, accounting for 18% (Emori & Gaynes, 1993).In England 100,000 cases of HAI are estimated to cost the National Health Service a minimum of £1 billion per year with more than 5,000 attributable deaths annually (Mayors, 2000).HAI is well documented in high-income countries, with major initiatives promoting cleanliness among hospital staff and visitors. However, despite many African hospitals being severely overcrowded and suffering frequent shortages of basic supplies such as running water and clean environment, scant data exist on the

number of hospital-acquired infections and the attendant costs to the economy in the region.

Respiratory Syncitial Virus (RSV) is an important aetiological agent of respiratory system diseases. It is the principal cause of acute lower respiratory tract infection (ALRI), causing annual winter epidemics that frequently challenge health resources (Avendano *et al*. 2003). In the U.S. alone, 100,000 infants are hospitalized annually for RSV infection and is associated with 11,000 deaths annually (Hayden, 2006). A study conducted in Chile to assess the burden of respiratory viral infection in the community recorded a mean detection rate of RSV to be 29%, which decreased significantly with increasing age and a 2% mean burden for hospitalization annually (Avendano *et al*. 2003).

The data presented here is for a study conducted on health-care associated respiratory syncytial virus in three public hospitals in Kenya using molecular genetics approach.

1.2 Problem Statement

Respiratory infections acquired during hospitalization have been documented to be a public health problem in Kenya with incidence of 9.2 infections per 10,000 patients and Respiratory syncytial virus (RSV) was one of the leading cause of infections (Ndegwa *et al*, 2014). RSV has been reported to be the leading cause of deaths among children under 2 years of age (Hall,2000) and it has also been shown to cause high incidence of respiratory infections in hospitalized patients (Nokes *et al*, 2009). Community based RSV surveillance has been conducted in Kenya to understand the epidemiology of the virus in the region and reported a high prevalence (17%) in children <5 years than in those >5 years (Bigogo *et al*, 2013).Extensive studies on RSV characterization have also been conducted in the coastal region with aim of understanding the circulating strains in the community (Agoti *et al*, 2014).

1.3 Justification

Many efforts have concentrated on the epidemiology and seasonality of RSV in Kenya but there is limited data describing RSV as a health-care associated respiratory infection causing virus. There is also limited data on the presentation of

the different subtypes/genotypes among the infected patients. This study was design to determine the transmission dynamics of RSV in the three referral hospitals of study by determining the characteristics of the circulating strains as a prerequisite to infection control.

1.4 Research Questions

This study aims to address the following main questions:

i) What are the age of patients who are commonly affected by HAI RSV in the three referral hospitals and the wards where most of the patients are drawn from?

ii) What are the most common subtypes observed during the study period?

iii) Was there any RSV Subtype/genotype relatedness over the study period?

1.5 Objectives

1.5.1 General Objective

To characterize hospital acquired respiratory syncytial virus from patients admitted in three referral hospitals; Kenyatta National Hospital, New Nyanza provincial General Hospital and Mbagathi District Hospital, using molecular methods.

1.5.2 Specific Objectives

1. To determine the demographic characteristics of patients with RSV in the three referral hospitals during the study period.

2. To determine the RSV subtypes/genotypes involved in HAI among patients admitted at the three referral hospitals during the study period.

   To determine the temporal and genetic relationships of RSV strains isolated from individuals who developed illness while admitted at the three referral hospitals.

CHAPTER TWO

LITERATURE REVIEW

2.1 Hospital Acquired Respiratory Infection

Respiratory infections represent the second most common cause of hospital acquired infections (WHO, 2000). Respiratory Hospital Acquired Infections (rHAI) are caused by bacterial, viral and fungal pathogens. These pathogens may originate from the environment, contaminated hospital equipment, health care workers or other patients. Pneumonia remains a major global public health problem and an important focus for scientific study. The World Health Organization estimates that lower respiratory tract infections cause nearly 4 million deaths per year, a rate of more than 60 deaths per 100,000 population (WHO, 2004). In the United States, nosocomial respiratory infections are major causes of excessive morbidity and mortality in the hospitals, affecting an estimated five to ten of 1000 patients (Hall *et al*, 2009). A study conducted in a Moroccan hospital revealed HAI prevalence of 10.3 with respiratory infections being classified as one of the leading causes of HAI (Razine, *et al*. 2012).

In Kenya, a study on viral etiologies of severe pneumonia among Kenyan infants and children conducted in Kilifi District Hospital indicated that RSV was the predominant pathogen (Berkley *et al.,* 2010) and was highest in children under 6 years with a prevalence of 15% (Nokes *et al,* 2008) Respiratory infections have also been associated with high rate of illness in two refugee camps, Dadaab and Kakuma in Kenya, RSV and Adenovirus have been found to be the most common cause of infection (Ahmed *et al.,* 2012). A study conducted in western Kenya in Nyanza province indicated that a significant burden of disease in children was caused by Influenza A virus (Waitumbi *et al.,* 2010). RSV has also been described as the most common cause of respiratory infection in Kilifi and the majority of this morbidity occurs in late infancy and early childhood (Nokes *et al*,2008). The findings of the different studies in Kenya indicate that a respiratory infection especially RSV is of public health concern and affects especially children.

2.2 Viral aetiologies of respiratory infections

Viruses are important causes of lower respiratory tract infection (LRTI) in developing countries (Weber *et al.,* 1998). The most common cause of viral LRTI are RNA viruses; Respiratory Syncytial Virus (RSV), human parainfluenza virus (PIV), influenza virus, and human metapneumovirus (hMPV) with Adenovirus being the exceptional DNA virus that commonly causes respiratory infections (Weber *et al.,* 1998).

2.2.1 Respiratory Syncytial Virus (RSV)

Respiratory syncytial virus was originally identified in 1956 from a laboratory chimpanzee with a respiratory illness and was later discovered to be of human origin (Collins & Graham, 2008). The virus is a linear single stranded negative-sense RNA consisting of 15,191 base pairs and the genome encodes for 11 proteins including structural and non-structural (Collins & Graham 2008).

RSV is classified into two subtypes, A and B based on their serological differences, in viral envelope protein (Collins & Graham 2008). Most studies have found RSV subtype A to be causing greater disease severity than RSV-B. RSV has three envelope proteins referred to as the small hydrophobic protein (SH), G protein, and F protein. F protein promotes fusion of the viral and cell membranes, allowing penetration of the viral ribonucleoprotein into the cell cytoplasm and G protein mediates virus binding to the cell receptor (Van Den Hoogen *et al.,* 2002). G is known to be the most variable protein in this virus, the nucleocapsid (N) gene showing the least variability and the small hydrophobic (SH) gene being intermediate.

The virus attaches to the cell of the nasal mucosa and upper respiratory tract via the G protein and the F protein allows fusion of the viral envelope with the host cell plasma membrane. Infected cells may undergo necrosis and syncytia form through cell-cell fusion which leads to spread from upper to lower respiratory tract (Van Den Hoogen *et al.,* 2002).

The Respiratory Syncytial Virus is a major cause of respiratory illness in all ages and it is the most common cause of nosocomial infection in paediatric wards with up

to 45% of contacts acquiring infections .The virus is a major cause of morbidity in infants and young children and produces cough rhinitis and mild fever over a period of four to five days. In adults, it tends to cause mild cold symptoms, in school-aged children it can cause a cold and bronchial cough while in infants and toddlers it can cause bronchitis or pneumonia. The virus has also been found to be a frequent cause of middle ear infections (otitis media) in preschool children (Hall, 2000).

Transmission may occur via ungloved or unwashed hands of the healthcare worker or relative of the patient or through contamination of the environment by coughing or sneezing. RSV can persist on skin or porous surfaces such as gowns or tissue paper for up to 30 minutes and up to 6 hours on non-porous surfaces like gloves. (Hall *et al.,* 1980)

RSV has a global distribution. In the U.S, reports reveal that cross-infection in pediatric wards is a common problem with over 40% children becoming infected if hospitalized during the winter for more than 7 days and each year approximately 50% of the paediatric staff acquire the infection (Hall, 2000). A recent study conducted between 2000 to 2004 documents the average rate of RSV associated hospitalization in USA to be 3 per1000 children under the age of 5 years and 17 per 1000 children under the age of 6 months. The authors further associated the infection with substantial morbidity in both in-patients and out-patients in the US. (Hall, 2000). In one outbreak in Toronto, Canada, three distinct sources of RSV with transmission of each strain within the ward were identified resulting in death of the eight patients (Mazuli *et al.,*1999). Immunocompromised adults are also at a risk for serious illness due to RSV infection therefore transmission of RSV within the hospital setting should be a concern with regard to the adult population. A study conducted among hospitalized children in Ohio, USA between 1985 to1988 indicated that both subtypes circulated and caused the same spectrum of diseases with type A being the most common. Subtype A was also found to be the most predominant in the community (Stark *et al.,* 1991).

A study in rural Kenya identified RSV as a major cause of respiratory infections in the community with the majority of the morbidity occurring in late infancy and early childhood and a rapid loss of immunity upon symptomatic re-infection (Nokes *et al,* 2008).

2.2.2 Health-care Associated Respiratory Syncytial Virus

Nosocomial outbreak of RSV was first described 1941 and it was associated with a high mortality despite having unexpected clinical manifestation (Adams, 1941).In 1937 outbreaks were identified in the nurseries of two of 2 Minneapolis hospitals where Thirty-two infants, aged between one to three months developed pneumonia, and 28% died All of the infants who died had same clinical presentation. Two decades later, a similar outbreak was experienced among infants in a hospital and it was identified to have been caused by the newly discovered RSV (Adams *et el,* 1963). Respiratory syncytial virus has an atypical presentation that can cause failure to recognize an outbreak in the wards hence facilitate its spread. Medical personnel are highly likely to be an important vector for transmission because the infection presents as a common cold or with flu like symptoms and in most cases most adults do not seek treatment for such symptoms because they are considered 'mild'.Even though the great majority of RSV infections in medical personnel are asymptomatic, they are accompanied by high viral shedding making it easy for the medical personnel to spread the infection to the patients in the wards (Hall , 2000) The immunocompromised and old patients are also at risk of acquiring nosocomial RSV. The virus can be introduced unrecognized into the ward by a family member or healthcare worker and cause an outbreak in the wards taking advantage of the patients reduced immunity. The virus has been documented to be the most common pathogen among institutionalized populations of older adults; accounting for 27% of all respiratory illnesses tested (Falsey *et al.,* 1992) and has also been documented to be an opportunistic infection in patients with underlying infections (Couch *et al.,* 1997). A survey conducted among the adult cancer patients with respiratory infections at M.D Cancer Center in Houston, identified RSV as the major cause of respiratory infection (Couch *et al.*, 1997). Hence it is of public health concern.

2.2.3 Factors Predisposing a Patient to Health-care Associated RSV Infection

Risks factors for the acquisition of infections nosocomial include; age, immune status, length of hospital stay, Iatrogenesis includes pathogens on the hands of medical personnel as a result of inadequate standards of proper hygiene and lack of use of personal protective equipment, for example failure to wash hands after handling patients and change gloves between patients (Weinstein, 1991).

Invasive procedures include extended ventilation, urine catheterization and intravenous lines. These equipment when used without proper hygiene, training or laboratory support lead to spread of infections from patient to patient . Organizational risk factors can be as a result of contaminated air conditioning, poor ventilation, and overcrowding of patients in the wards.

2.2.4 Characteristics of RSV that enhances its nosocomial transmission.

The capability of RSV becoming a major nosocomial pathogen is due to its several characteristics; unlike most bacterial agents that cause nosocomial infections, persons of all ages both healthy and with underlying conditions are potentially susceptible to RSV infections, RSV outbreaks occurs annually and the infections spreads at a very first rate therefore can easily be transferred from the community to the healthcare set up. After the first infection in infants, there is normally incomplete immunity and this results in repeated infections throughout life, often within short intervals, even weeks or months (Hall *et al*, 1991).In children, RSV in secretions tends to shed for long and at high titres hence increasing the chances of spreading the infection and may remain contagious in the environment for periods long enough for inadvertent transmission on hands and fomites (Hall *et al*, 1980). Secretions from young children frequently contaminate the objects surrounding their beds, such as bed railings, table tops, and toys, and may remain viable and contagious for hours, depending on the type of surface, environmental temperature, and humidity. low humidity enhances the duration of RSV's viability, allowing it to survive on nonporous surfaces for 6–12 h or more (Hall *et al*, 1980)

2.2.5 Main Routes of Transmission of Hospital Acquired Infection

Contact transmission is an important and frequent mode of transmission of infections. During routine patient care, the personnel can increase contamination by handling multiple patients without changing gloves or might not use hand gloves at all. Contact transmission can be direct or indirect (Siegel *et al.,* 2007). Direct transmission occurs when microorganisms are transferred from one infected person to another person without a contaminated intermediate object or person while indirect transmission involves the transfer of an infectious agent through a contaminated intermediate object or person (Siegel *et al.,* 2007).

Droplet transmission occurs when respiratory droplets are generated from one infected person and deposited on the body of an uninfected person (Siegel *et al.,* 2007). This occurs mainly during coughing, sneezing or even talking .Infection transmission can also occur when small particle residue of evaporated droplets containing microorganisms remain suspended in the air and are then inhaled by a susceptible host within the same room or over a long distance from the source patient (Bloch *et al.,* 1985).
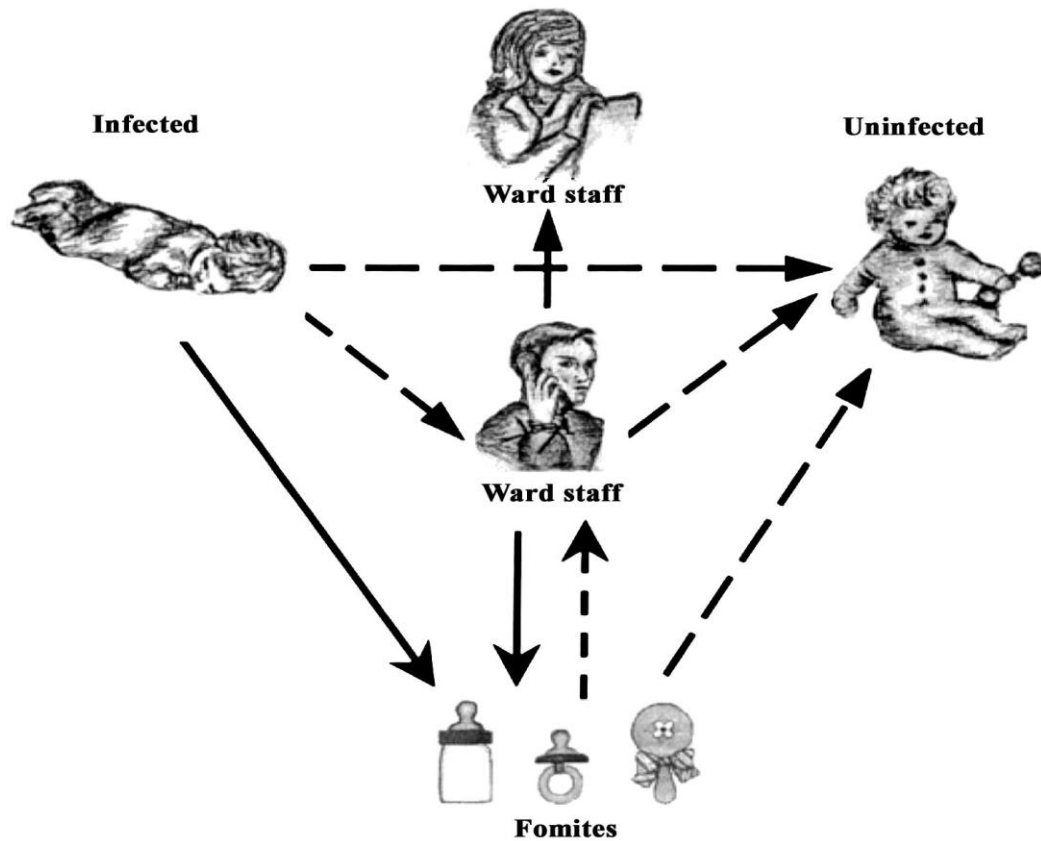
Figure 1. Modes of transmission of RSV infection from an infected patient to uninfected patient; Modes of nosocomial spread of respiratory syncytial virus. *Dashed lines*, routes of transmission most likely to be interrupted by infection control; *solid lines*, routes of transmission unlikely to be affected appreciably by infection control procedures.

2.2.6 Control and treatment of RSV

Active surveillance of all symptomatic patients and healthcare workers especially during RSV season is the most convenient way for identifying outbreaks (Kassis *et al.,* 2010). Personal protective equipment like gloves, masks should be used by healthcare workers when handling patients, this should be used in conjunction with the simple and inexpensive preventive measure of hand washing and strict adherence to infection control measures.Intensive educational programs help increase awareness of RSV exposure among patients, caregivers, and family members of the infected patients (Forbes, 2008)

Cleaning of the hospital environment with a hospital grade detergent/disinfectant to both remove organic matter and inactivate infectious particles is recommended (Forgie & Marrie, 2009). Kassis *et al.* (2010), have demonstrated the use of palavizumab antibody in controlling the spread of nosocomial RSV. Palivizumab is a human antibody that specifically inhibits the A antigen site of the F protein of RSV A and B. Binding to the F proteins prevents cellular infection by preventing the viral membrane fusing with the respiratory epithelial cell membrane and prevents cell to cell spread of the virus which prevents formation of the syncyntia and release of inflammatory mediator in the lungs (Scott & Lamb, 1999). However the vaccine has been found to be too expensive for wide range use especially in developing countries.

Both supportive and antiviral therapy has been documented as a consideration for RSV infection management (Falsely & Walsh, 2000). Ribavirin is one of the proven agents but its use in infants is still controversial since randomized trials have not demonstrated any success in efficacy (Falsey & Walsh, 2000).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Design

This was a laboratory based descriptive study characterizing RSV acquired in the hospital. The study used archived nasopharyngeal and oro-pharyngeal samples collected during the surveillance study from September 2009 to July 2011, the study samples were tested in the Centres for Disease Control laboratories in KEMRI headquarters, Kenya.

3.2 Study Sites

This was a sub study of an ongoing hospital acquired infection surveillance study that is being conducted by KEMRI/CDC under the Infection Control Programme (ERC1571). The samples were collected from Kenyatta National Hospital, New Nyanza Provincial General Hospital (Jaramogi Oginga Odinga Hospital)and Mbagathi District Hospital. Kenyatta National Hospital is a referral hospital located in Nairobi. It has 1800 beds with 89,000 admissions and 600,000 outpatient visits annually with a 7 day average length of hospital stay. It has several types of wards which include; medical, surgical, paediatric and special units.

New Nyanza Provincial General Hospital (NNPGH) is a provincial referral hospital located in Kisumu City, Kisumu County. It is the largest public hospital in the former Nyanza Province Kenya. It receives referrals from the western part of Kenya. It has 3000 beds, 18,300 admissions and 13,306 outpatient visits annually.

Mbagathi District Hospital is located off Mbagathi road in Langata district, Nairobi county. The hospital serves the general population in Nairobi and is the largest TB hospital in Nairobi therefore receiving a vast number of pneumonia cases; it also called infectious disease hospital. It has about 600 beds, with approximately 800 admissions monthly and about 8,000 outpatient visits.

3.3 Study Population

The study population consisted of all patients who had been admitted in the paediatrics wards, general wards, intensive care unit and the special units with other underlying infections other than respiratory infections.

The population of interest was the RSV positive cases collected from these patients between September 2009 to July 2011.

3.4 Sample Size Determination

The study used a census of all 286 samples collected these were archived samples used in a previous study (ERC 1571).census eliminates sampling error and with a desirable level of precision provides data on all the individuals in the population (Glen, 1992).

3.5 Laboratory Procedures

3.5.1 Ribonucleic Acid Extraction

RNA was extracted from all 286 samples by QIAamp® Viral RNA Kit and the cDNA synthesis was carried using an OmniscriptTM kit according to the manufacturer's instructions (Qiagen, Hilden, Germany).

The samples were removed from the freezer and left to thaw at 40 C then allocated processing identification number. They were vortexed to ensure homogenization then 140 μl was added to the lysis buffer AVL-carrier RNA solution in the ratio 1:4, mixed by vortexing for 15 seconds to ensure efficient lysis then incubated at room temperature for 10 minutes.

Ethanol was then added to each sample and mixed thoroughly. Ethanol forces the precipitation of nucleic acids out of solution. After precipitation the nucleic acids were then separated from the rest of the solution by centrifugation. The pellet was washed in cold 70% ethanol, to ensure that the nucleic acid is clean from any residual salt. After a further centrifugation step the ethanol was removed, and the

nucleic acid pellet allowed to dry before being resuspended in clean elution buffer. The RNA was then stored at -80°C freezer.

3.5.2 Nucleic Acid Amplification

RT-PCR probe Analysis using the Agpath PCR mastermix kit

Reagents

- Buffer

- Forward primers

- Reverse Primers

- Probes

- Enzyme

- Positive control materials

- Nuclease free water

Equipment

- Vortex

- RT-PCR detection system (Applied Biosystems 7500)

- Biosafety cabinet

Primers and probes

All primers and probes were left to thaw at 4°C then vortexed to ensure homogenization

Reaction set up

The mastermix was prepared using Agpath ID One step RT-PCR kit in accordance to the number of reactions required. This is calculated for each reagent as provided in the Agpath PCR mastermix table for standardized primers. Each target was tested by

separate primers and probe sets; RSV and RNase P (RNP) which targets the human RNase which is used as an internal positive control for human nucleic acid.

The nucleic acid amplification was done using the 7500 FAST real-time polymerase chain reaction platform (ApliedBiosystems), where all samples were screened for respiratory syncytial virus alongside other viruses(Influenza A, influenza B, human metapneumovirus, Parainfluenza virus 1,parainfluenza 2 and parainfluenza 3).

The master mix was prepared with RSV specific primers with a working concentration of 30pmol/μl, Forward (GGC AAA TAT GGA AAC ATA CGT GAA), Reverse (TCT TTT TCT AGG ACA TTG TAT TGA AGAG) together with the specific probes (5'CTGTG TAT GTG GAG CCT TCG TGA AGC T'3) using AgPath ID One-step RT-PCR. The kit contains buffer and the enzymes to provide optimal reaction and activation environment respectively. The 5 μl RNA template was added to 20 μl of the master mix, and then loaded to the 7500 Fast Real-Time PCR platform set at 45 cycles with suitable optimal cycling conditions; Reverse transcriptase stage was set at 45 ^{o}C for 10 minutes, where the RNA was first converted to cDNA, taq polymerase activation stage was set at 95 ^{o}C for 10 minutes, denaturation at 95 ^{o}C for 15 seconds then primer anealing and elongation stage was at 55 ^{o}C for one minute.DNA amplifications plots were then viewed on the PCR machine to identify the positive samples, these were formed as a result of the SYBR green dye intercalating in the DNA helix then fluorescing to form the curves. As the PCR product increases, the amount of fluorescence also increases and is quantified once each cycle is complete. The cycle at which the fluorescence exceeds a detection threshold (cycle threshold) correlates with the quantity of cDNA molecules. The cycle threshold values were used to determine RSV positive samples. Ct Value (cycle threshold) is defined as the amount of cycles required for the fluorescence signal to cross the threshold and exceed background level. Ct levels are inversely proportional to the amount of target nucleic acids in a sample. The test was set at 45 cycles of amplification.

Ct < 30 is a strong positive reaction indicative of abundant target nucleic acid in the sample

Ct of 31-37 is a positive reaction indicative of moderate amounts of target nucleic acid

Ct of 38-45 is a weak reaction indicative of minimal amounts of target nucleic acid which could represent an infection state or environmental contamination.

Only specimens with a cycle threshold (CT) value of 30 or lower were used as it had previously been observed in the laboratory that there was limited success with sequencing specimens that had CT values >30 for RSV. Group specific PCR was carried out to classify the RSV positive samples into groups A and B.

Amplifications were carried out as nested RT-PCR, the first round amplification was performed using Qiagen one-step RT-PCR kit (QIAGEN Ltd) with the forward primer AG20 (5'-GGGGCAAATGCAAACATGTCC–3') and the reverse primer F164 (GTTATGACACTGGTATACCAACC).

Thermocycling conditions were set as follows: Reverse transcription stage for 30 min at $50^{o}C$, 15 minute at $95^{o}C$ denaturation, annealing for 1 minute at 95 ^{o}C, the elongation stage was for 1 minute at 55 ^{o}C and then cooling for 1 minute at 72 ^{o}C. The process occurred in 40 cycles.

For the second round PCR the forward primer BG10 (5' GCAATGATAATCTCAACCTC-3) and reverse primer F1 (CAACTCCATTGTTATTTGCC) were used and the thermocyclic conditions were; Reverse transcription for 2 minutes at $95^{o}C$, followed by denaturation for 1 minute at $95^{o}C$ then annealing for 1 minute at 50 ^{o}C and lastly extension for 2 minute at $72^{o}C$.(appendix ii) (Agoti *et al* 2012). To confirm success in amplification, the product was visualized by Ethidium bromide stained gel electrophoresis on a 2% agarose gel (Appendix iii)

3.5.3 Gel Electrophoresis procedure

1. Preparing 0.5 TBE (Tris base/Boric acid/EDTA) buffer

The following was mixed in a 2 L flask:

    -108 g Tris base

    -55 g Boric acid

    -40 ml 0.5 M EDTA pH 8.0

    -Distilled water was then topped up to 2 L

The mixture was then placed on a magnetic stirrer until completely dissolved to give a 5x stock.

The stock was diluted by 10 in distilled water to give the working concentration.

2. 2% Gel preparation

    -Agarose

    -Ethidium bromide

    -0.5 TBE buffer

4 grams of agarose was added in 200ml of 0.5% TBE, heated in a microwave to dissolve then left to cool.5µl of ethidium bromide was added to enable visualization of the DNA.

The gel was then poured into the electrophoresis tank fitted with combs. Once it cooled, the combs were carefully removed and samples loaded into the hole. The sample is mixed with the loading dye to increase the density and coalesce the sample.

The gel was placed in the electrophoresis chamber with the wells closest to the negative (black) electrode. Then the salt solution was gently added into the chamber to fill up above the gel. Then connected to the power supply. After the run was complete the power supply was switched off and the gel removed and photo taken to analyse the results of the electrophoresis.

3.5.4 G gene product purification using GFX purification kit

The product is purified to remove residual PCR primers and unincorporated nucleotides. This was done in four stages;

1. sample capture -where buffer type 3 was added to the sample to denature the proteins.

2. Sample binding - the sample and buffer mixture was added to the illustra GFX spin column to allow DNA to bind to the membrane.

3. Wash and dry- buffer type one was added in the spin column to remove salts and other contaminants from the membrane bound DNA..The columns were then put in a centrifuge and spun to dry up.

4. Elution – Elution buffer type 6 was added into the spin columns to elute the samples

3.5.5 Cycle sequencing reactions

The sequencing master mix was prepared using the dye terminator, buffer, primers and nuclease free water then loaded onto the microfuge plate. The plate was then loaded to the thermal cycler machine.The thermocyclic conditions were set, rapid thermal ramp at 96 °C for 30 seconds, 50 °C for 15 seconds and the final ramp at 60 °C for 4 minutes. This was repeated for 25 cycles

Precipitation was done to remove any unused material and Hi di was added to dissolve the DNA (Appendix iv). The microfuge plate was removed from the thermal cycler then 1µl on 25Nm Ethylenediaminetetraacetic acid (EDTA), then 1µl of 3M sodium acetate and 25µl of 100% ethanol was added to each well. The plate was sealed and incubated at room temperature for 15 minutes then drained all the supernatant.100µl was added then spun at maximum speed fo 5 minutes while maintained at 20°C. The supernant was drained off then let to totally dry up[for thirty minutes.Hi di formamide was added into each well of the plate to resuspend the samples before electrokinetic injection on capillary electrophoresis systems.The

plate was then loaded to the thermocycler for 3 minutes at 95°C for denaturation reaction to occur. Finally the plate was loaded to the 3130xl sequencing machine.

Table 1: List of primers used in PCR and Nucleotide sequencing (Agoti et al.,2012)

RSV A sequencing primers	RSV B sequencing primers
BG10(GCAATGATAATCTCAACCTC)	10 (GCAATGATAATCTCAACCTC)
F1 (CAACTCCATTGTTATTTGCC)	F1 (CAACTCCATTGTTATTTGCC)
523F(5'ATATGCAGCAACAATCCAAC -3')	533F(5'TGTAGTATATGTGGCAACAA - 3')
523R (5'GTTGGATTGTTGCTGCATAT -3')	533R(5' TTGTTGCCACATATACTACA - 3')

3.5.6 Sequence analysis

Contig assembly was done in sequencher version 5.10, (Gene codes corporation, USA) (Agoti *et al*, 2012). Here a set of overlapping DNA segments that represent a consensus region of DNA are assembled into contigs and a direct comparison of trace date with nucleotide data made possible. This allows for proof reading and base editing. Sequence alignments were done in BioEdit (Version 7.2.0) in aligning, other express sequence tags are added to a sequence that is not full length and mismatches are identified for single nucleotide porlymorphism. Sites that share a common evolutionary history are also identified hence provide a measure of biological relatedness in between neuclotides therefore can be able to tell biological inferences regarding structural, functional and evolutionary relationships.

Phylogenetic trees were generated in MEGA 5 using the neighbor joining methods (Tamura *et al*, 2011). The appropriate model of evolution was determined in jmodeltest (Posada, 2008). The model allows for the calculation of probabilities of change between nucleotides along the branches of a phylogenetic tree. Models of

nucleotide substitution allow for the calculation of probabilities of change between nucleotides along the branches of a phylogenetic tree.

Sequences from GenBank representative of all known RSV genotypes were included in the alignments to genotype the strains we identified. The robustness of the tree branching patterns was evaluated by bootstrapping with 1000 iterations. The trees were draContig assembly was done in sequencher version 5.10, (Gene codes corporation, USA) (Agoti *et al*, 2012). Here a set of overlapping DNA segments that represent a consensus region of   DNA are assembled into contigs and a direct comparison of trace date with nucleotide data made possible. This allows for proof reading and base editing. Sequence alignments were done in BioEdit (Version 7.2.0) in aligning, other express sequence tags are added to a sequence that is not full length and mismatches are identified for single nucleotide porlymorphism. Sites that share a common evolutionary history are also identified hence provide a measure of biological relatedness in between neuclotides therefore can be able to tell biological inferences regarding structural, functional and evolutionary relationships.

Phylogenetic trees were generated in MEGA 5 using the neighbor joining methods (Tamura *et al*, 2011). The appropriate model of evolution was determined in jmodeltest (Posada, 2008). The model allows for the calculation of probabilities of change between nucleotides along the branches of a phylogenetic tree. Models of nucleotide substitution allow for the calculation of probabilities of change between nucleotides along the branches of a phylogenetic tree.

Sequences from GenBank   representative of all known RSV genotypes were included in the alignments to genotype the strains we identified. The robustness of the tree branching patterns was evaluated by bootstrapping with 1000 iterations. The trees were drawn to scale with 0.01 nucleotide substitution.

3.5.7 Data Management

3.5.7.1 Data Collection Tools

Identifier information was abstracted from the questionnaire which had been administered to the respondents at the three hospitals. The data was delinked to remove the patient identifiers before analysis.

3.5.7.2 Data Storage

All the data collected including patient information and laboratory results were entered into a Microsoft excel database.

3.5.7.3 Data Processing and analysis

All patient information was abstraced from the questionnaires and entered into a Microsoft excel database. The data was cleaned then merged to the laboratory results using their unique identification numbers. The analysis was divided into three stages, namely the univariable, bivariable and multivariable stages. The univariable analysis stage consisted of frequency tables/values for the categorical study variables and the outcome (s) of interest. The bivariable stage was used to test the presence of an association between categorical study variables and RSV positivity and the statistical significance of the association. This was achieved using a simple binary logistic regression model with RSV positivity being the outcome of interest and modelled against each of the independent study variables. The multivariable analysis stage was also used to test the presence/absence of an association between categorical study variables and the outcome(s) of interest and the statistical significance of the association in a single regression model. The regression model of choice was a multiple binary logistic regression model with RSV positivity being the outcome of interest and modelled against all the significant independent variables in the study.

The results of the data analysis were presented in narrative and tabular forms.

3.5.8 Ethical Clearance

Samples from human participants used for this study were collected under protocol SSC No. 1571, using informed consent documents reviewed and approved by KEMRI Scientific Steering Committee and National Ethical Review Committee. Clearance for this sub-study was sought from the KEMRI Scientific Steering Committee and the KEMRI National Ethical Review Committee SSC number 2571 (appendix v and vi)

Consent to store aliquots of the samples for further analysis (SSC No. 1571) was sought from the participants prior to sample collection and the minors were assented for by parents or gurdians.

CHAPTER FOUR

RESULTS

4.1Demographic characteristics of patients with RSV

Table 2: Demographic characteristics of the study population (n = 248)

Group	Characteristics	n (%)
Gender	Male	150 (60.5)
	Female	98 (39.5)
Age Group	Adult	104 (41.9)
	Child	144 (58.1)
Hospital	Kenyatta National Hospital (KNH)	158 (63.7)
	Mbagathi District Hospital (MDH)	53 (21.4)
	New Nyanza Provincial General Hospital (NNPGH)	37 (14.9)
Wards	Burns	5 (2.0)
	Intensive Care Unit (ICU)	59 (23.8)
	Medical	30 (12.1)
	Paediatrics	124 (50.0)
	Special	26 (10.5)
	Surgical	4 (1.6)

From September 2009 to July 2011 a total of 286 samples were collected from patients who had been admitted with other underlying infections at KNH, MDH and NNPGH and tested, 248 were able to be linked back to the patient demographic data. Among the 248 patients admitted, males constituted 60.5 % (n=150) of the number, while children comprised slightly more than half 58.1% (n=144) of the patients admitted. Amongst the hospitals more than two thirds of the patients, 63.7% (n=158) were admitted at Kenyatta National Hospital.

Thirty seven patient specimens (14.9%) out of the 248 tested positive for RSV by routine real time PCR viral screening with 60 % of them being from male patients.

Majority (86.5%) of the RSV positive patient specimens were from children, while also slightly more than half of them were from patients admitted at Kenyatta National Hospital. Amongst the wards, the paediatrics ward contributed the vast majority (81.1%) of the RSV positive patient specimens.

Table 3: Demographic Characteristics and RSV positivity (n = 37)

	RSV Subtypes				
Characteristics	**Pos** **Total=37** **n (%)**	**A** **Total=13** **n (%)**	**B** **Total=6** **n (%)**	**AB** **Total=1** **n (%)**	**NonSubtype** **Total=17** **n (%)**
Gender					
Male	22 (59.5)	8 (61.5)	3 (50.0)	1 (100.0)	10 (58.8)
Female	15 (40.5)	5 (38.5)	3 (50.0)	0 (0.0)	7 (41.2)
Age Group					
Adult	5 (13.5)	0 (0.0)	0 (0.0)	0 (100.0)	5 (29.4)
Child	32 (86.5)	13 (100.0)	6 (100.0)	1 (100.0)	12 (70.6)
Hospital					
KNH	22 (59.5)	7 (53.8)	4 (66.7)	1 (100.0)	10 (58.8)
MDH	9 (24.3)	3 (23.1)	1 (16.7)	0 (0.0)	5 (29.4)
NNPGH	6 (16.2)	3 (23.1)	1 (16.7)	0 (0.0)	2 (11.8)
Wards					
Burns	1 (2.7)	1 (7.7)	0 (0.0)	0 (0.0)	0 (0.0)
ICU	4 (10.8)	0 (0.0)	0 (0.0)	0 (0.0)	4 (23.5)
Medical	1 (2.7)	0 (0.0)	0 (0.0)	0 (0.0)	1 (5.9)
Paediatrics	30 (81.1)	12 (92.3)	6 (100.0)	1 (100.0)	11 (64.7)
Special	1 (2.7)	0 (0.0)	0 (0.0)	0 (0.0)	1 (5.9)

From the thirty seven (37) RSV positive patient specimens, 13 (35.1%) were subtype A, six (6) were subtype B, one (1) was subtype AB and seventeen 17 (45.9%) were unable to subtype. Majority of the thirteen (13) subtype A, RSV positive patient specimens were from males (n=8, 61.5%) among gender, children (n=13, 100.0%) among the age groups, KNH (n=7, 53.8%) among the hospitals and paediatrics ward (n=12, 92.3%) among the wards.

Majority of the six (6) subtype A, RSV positive patient specimens were from children (n=6, 100.0%) among the age groups, KNH (n=4, 66.7%) among the hospitals and paediatrics ward (n=6, 100.0%) among the wards.

From the seventeen (17) RSV positive patient specimens that were unable to subtype, majority were from males (n=10, 58.8%) among gender, children (n=12, 70.6%) among the age groups, KNH (n=10, 58.8%) among the hospitals and paediatrics ward (n=11, 64.7%) among the wards

Table 4: Bivariable and Multivariable Analysis of Demographic Characteristics in relation to RSV Positivity

Group	Characteristics	Unadjusted Odds Ratios		Adjusted Odds Ratios	
		Odds Ratio	P-value	Odds Ratio	P-value
Gender	Male	Reference			
	Female	1.05	0.8901	_	_
Age Group	<2 years	Reference		Reference	
	2-4 years	0.73	0.5047	0.85	0.7342
	5+ years	0.13	<0.0001*	0.21	0.0117*
Hospital	KNH	Reference			
	MDH	1.26	0.5870	_	_
	NNPGH	1.20	0.7207		
Wards	Paediatrics	Reference		Reference	
	ICU	0.23	0.0081*	0.63	0.5002
	Others	0.15	0.0026*	0.37	0.1546

From (Table 4), it was seen that in the bivariable analysis, females patients were 5% more likely to become RSV positive as compared to their males counterparts, though this relationship was not statistically significant, (OR=1.051, p = 0.8901). Similarly from the table, it was seen that those patients admitted aged 5 years and above, were 87% less likely to become RSV positive as compared to those aged below 2 years; this relationship was statistically significant, (OR=0.132, p = <0.0001). Amongst the wards, patients admitted in the ICU and those admitted in other wards had statistically significant relationships with RSV positivity , with those patients admitted in the ICU having odds that were 77% lower than those in the paediatrics ward, (OR=0.228, p = 0.0081) while those in the other wards had odds that were 85% lower than those of those in the paediatrics ward, (OR=0.152, p = 0.0026).

Similarly from (Table 4), it was seen that in the multivariable analysis, it was seen that those patients admitted aged 5 years and above, were 79% less likely to become RSV positive as compared to those aged below 2 years; this relationship was statistically significant, (OR=0.21, p = <0.0117). While amongst the wards, patients admitted in the ICU and those admitted in other wards did not have statistically significant relationships with RSV positivity, with those patients admitted in the ICU having odds that were 77% lower than those in the paediatrics ward, (OR=0.63, p = 0.5002) while those in the other wards had odds that were 85% lower than those of those in the paediatrics ward, (OR=0.37, p = 0.1546).

4.2 Subtypes and Genotypes of RSV circulating in the three hospitals.

Multiplex PCR confirmed the presence of both RSV A and RSV B groups in all the three sites with RSV A being the predominant subtype (Table 1) 13(35%) were RSV A, 6 (16%) RSV B, 1 was AB and 17 neither typed for A nor B. Twenty out of thirty seven specimens gave cycle threshold value below thirty which was used as the cut-off mark for sequencing. Of the twenty samples that were sequenced, seventeen gave successful sequencing, 9/17 were from patients at KNH, 4/17 from patients at MDH and 4/17 were from patients at NNPGH. (See figure 1 below showing sample flow)
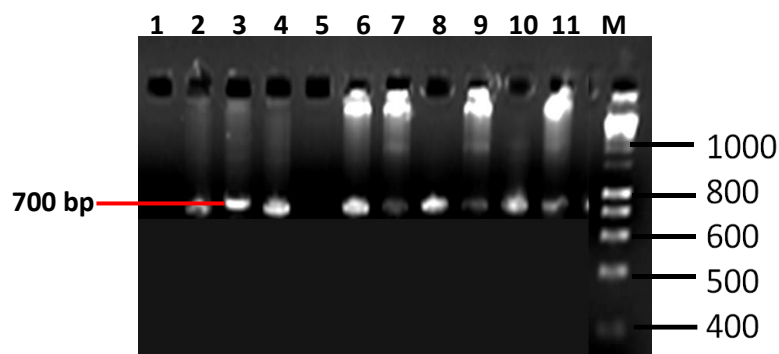
Gel 1. A representation of results by gel electrophoresis for RSV samples. Positive samples were detected at 700 bp. **Well 1:** negative control; **wells 2 – 11**: clinical materials; **M**: molecular weight marker (1000bp).

A representation of results by gel electrophoresis loaded with a 1000 base pair molecular weight marker indicates that the RSV sequences detected were at 700 base pair as expected.

17 samples sequenced successfully, nine samples (53%) were RSV A and 8(47%) were RSV B. The alignment and phylogenetic analysis of these sequences revealed that the majority of the Kenyan HRSV group A isolates belong to two genotypes, the NA 1 genotype and ON1 genotype with bootstrap support value of above 80% (Figure 2). ON 1 Genotype is characterized by a 72-nucleotide duplication in the attachment protein gene, has been detected in more than 10 countries since first identified in Ontario, Canada, in 2010 (Eshaghi *et al*, 2012) and was first detected in Kenya in 2012(Agoti *et al*, 2014).
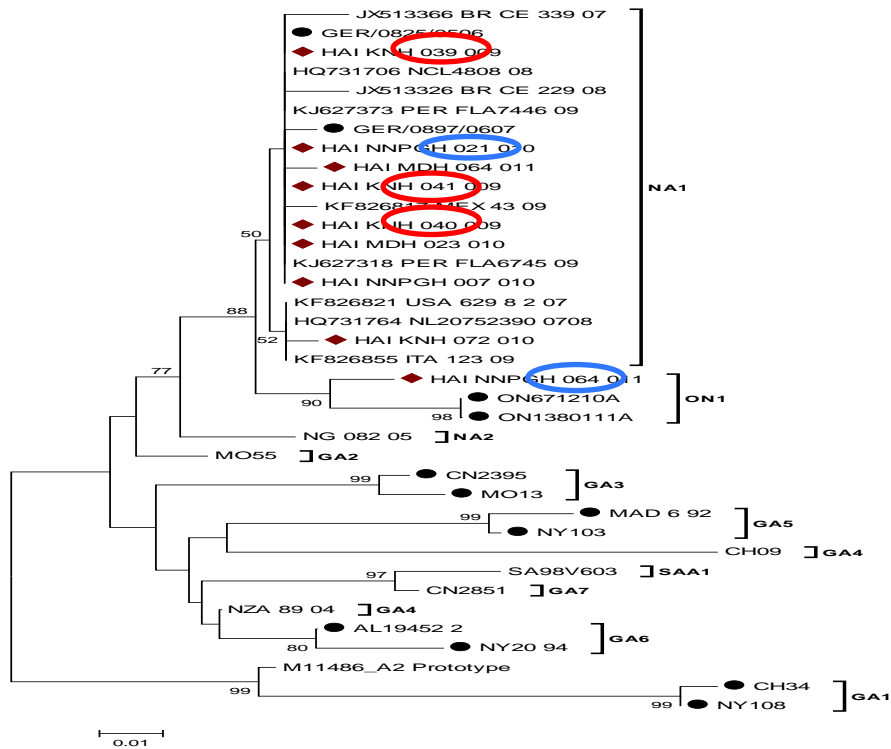
Figure 2: Phylogenetic tree of HRSV group A strains isolated in Kenya and reference strains retrieved from GenBank based on the hypervariable region of the G gene. The tree was generated by MEGA5 program using the neighbor-joining method.;**Diamonds** indicate strains obtained in this study, **blocked circles** indicate the reference strains from genebank, **red circles** indicates samples whose isolated DNA sequences are closely related and were from the same ward and the **blue circles** indicates isolated DNA sequences were of different genotype but were from the same ward.
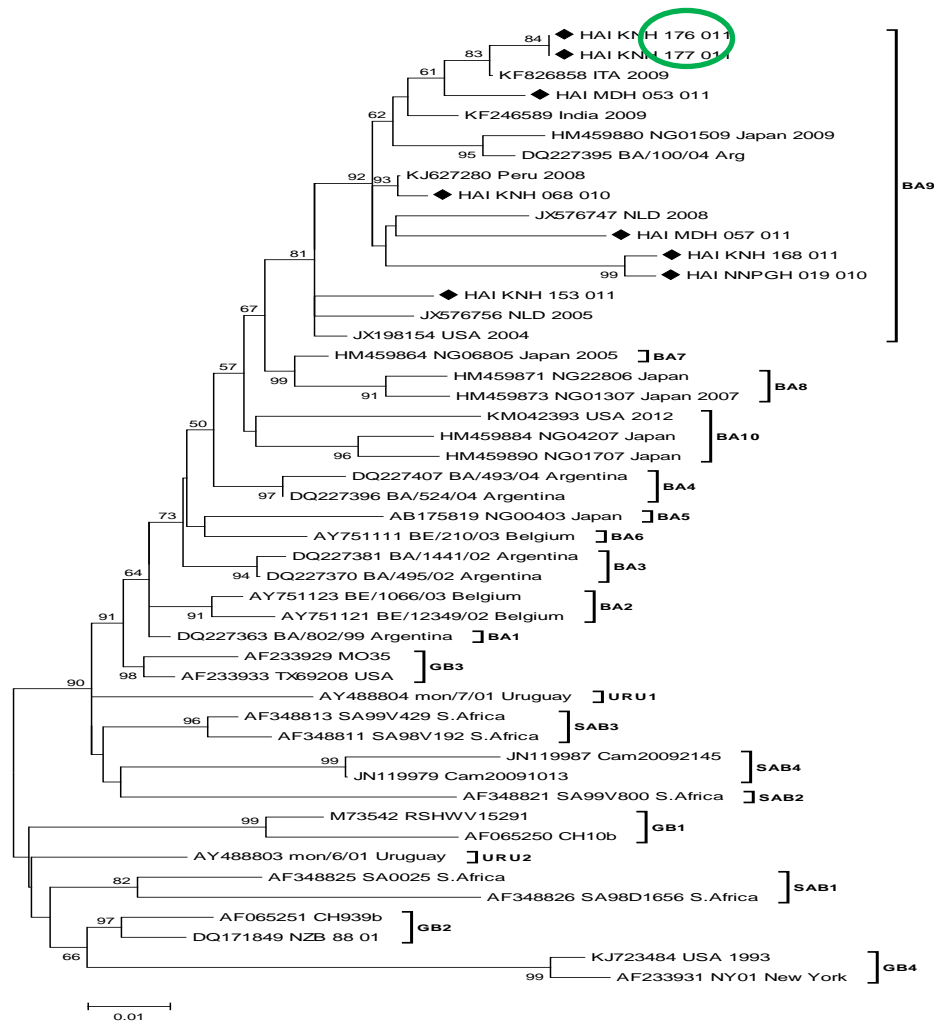
Figure 3: Phylogenetic tree of HRSV group B strains isolated in Kenya and reference strains retrieved from GenBank based on the hypervariable region of the G gene. The tree was generated by MEGA5 program using the neighbor-joining method. **Diamonds** indicate strains obtained from the study hospitals of study, **green circle** indicates isolated that were closely related and the samples were collected from patients in the same ward.

4.3 Temporal and Genetic Relationships of RSV Strains Isolated

In assessing the temporal relationship and genotype of patients, three patient samples from KNH had the same RSV A genotype with the same RSV detection date, though the patients had different lengths of hospital stay before the virus was detected. Two samples from MDH and one from NNPGH had the same genotype but they were all detected at different times of the study. Patient HAI NNPGH 007, KNH 040 , KNH 072 and NNPGH 021 acquired the infection long after admission. While patients HAI MDH 023 and MDH 064 acquired the infections within the same week of admission.

For the RSV B group, two samples HAI KNH 176 and 177 had the same genotype with the same date of virus detection. The two patients had different length of hospital stay but had both stayed in hospital for more than two months. It was also observed that all the patients except one HAINNPGH019 had been in hospital more than two weeks
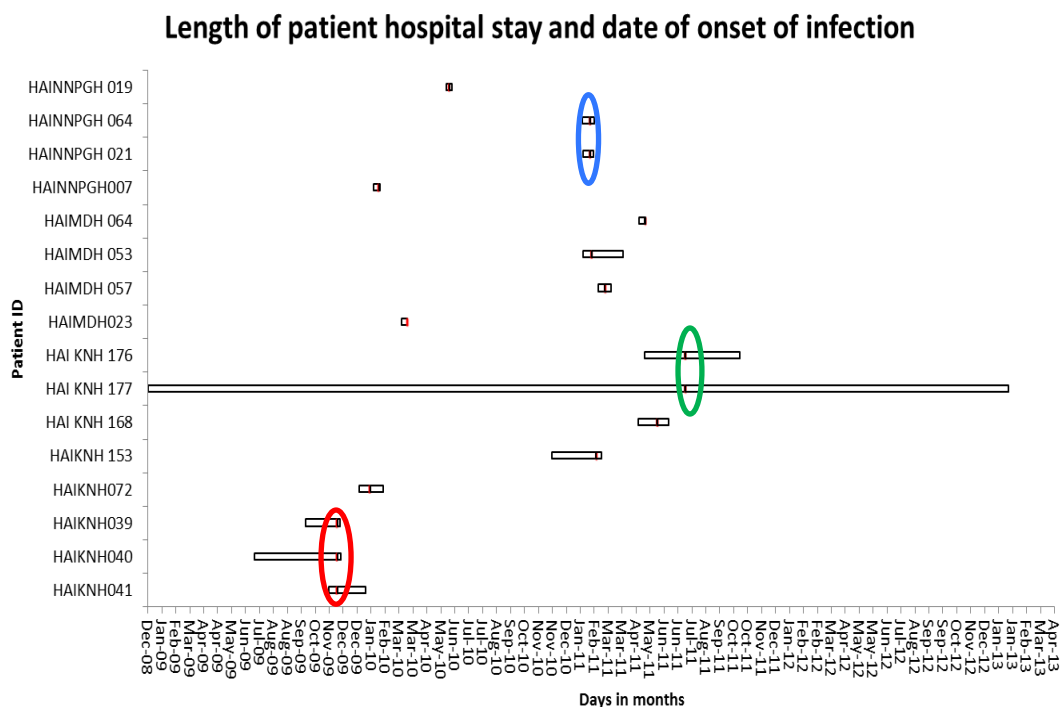
Figure 4: Length of patient hospital stay and date of onset of infection over the study period to show the temporal relationship of the genotypes isolate.

The bars represents the length of patient hospital stay, **red mark** on the bars represents date of onset of infection, **blue circle** represents patients from NNPGH who were in the same ward with same date of RSV A detection whose strain were genetically related. **Green circle** represents patients from KNH with same date of RSV B detection and with genetically related strains.

CHAPTER FIVE

DISCUSSION   CONCLUSION AND RECOMMENDATIONS

5.1 Discussion

RSV is recognized as the leading cause of respiratory tract illness in children and is a major nosocomial pathogen. We evaluated RSV genetic variability in nasopharyngeal and oropharyngeal samples taken from patients who had been admitted in three referral hospitals in Kenya 2009-2011. The patients had stayed in hospital for more than six days and had no influenza like symptoms at the time of admission suggesting that the virus was most likely acquired during their hospital stay.

In this study RSV was detected in 15% of samples collected from the patients. Majority of the patients infected by RSV were children below 2 years of age, they were likely more at risk than those aged 5 years. Similar findings have been reported by Nokes et al where the incidence and severity of RSV was found to be high among children less than 5 years of age. He reports the risk of RSV re-infection after the first infection to be high in children <6months followed by a decrease in risk with increase in age (Nokes *et al*, 2009). In adults, the most common manifestation after repeated exposure to RSV is that of a mild upper respiratory tract infection. Viral shedding subsequently occurs for a mean of 4–5 days (Hall, 2000), but may be prolonged for up to 120 days in immunocompromised patients.

Mbagathi District Hospital recorded the highest prevalence of RSV over the period but there was no significant difference in risk measure the risk of acquiring RSV was almost equally spread (MDH aOR =0.803, p=0.643; NNPGH aOR = 0.454, p=0.1432).

Genotypes identify differences between strains within group A or group B most likely to affect antigenic characteristics of these viruses, while subtypes identify viruses which are antigenically similar (Peret*et al*, 1998). Both RSV A and RSV B subtypes were observed to be in circulation during the study period with the majority being RSV A. co-circulation of groups A and B within the same population has been described during epidemic periods, and with different predominance patterns (Zhang

et al, 2010). Isolates collected from Kenyatta National, Jaramogi Oginga Odinga Teaching and Refferal Hospital and Mbagathi district hospitals were observed to be closely related because they all shared a common ancestral root despite their distances apart. This indicates that there was limited virus introduction within the hospitals but with wide scale spread. A recent study in a Kenyan refugee camp also reported co-circulation of both RSV A and B genotypes with RSV A predominating (Agoti *et al*, 2014).

Majority of the RSV A sequences from the three hospitals were categorized phylogenetically as NA1 genotypes closely related to isolates identified from Germany, Italy and USA. Only one RSV A isolate was classified as ON1, this genotype has also been detected in coastal parts of Kenya, (Agoti *et al*,2012) and it is characterized by a 72-nt duplication in the attachment protein gene.

All RSV B isolates were classified as BA9 genotype closely related to isolates from Japan, India, Italy and the USA. Observation of the emergence and rapid spread and diversification of RSV group B genotype BA (containing a 60-nt duplication) has improved our understanding of the scale of RSV spread and transmission in the world (Malero *et al*, 1997). Our findings regarding the ON1 genotype, which also possesses a large nucleotide duplication, closely relate to findings regarding the BA genotype and confirm that novel RSV strains do spread rapidly and widely.

RSV outbreaks occurs every year and affects both the healthy and individuals with underlying infections affecting patients of all ages and has been documented as the most frequent cause of nosocomial infection(Hall *et al*, 2000). Incomplete immunity to RSV results to repeated infections throughout life, this together with the fact that shedding of the virus in the respiratory secretions of young children tends to be for long periods and at high titer (Hall *et al*, 2000), facilitates it's nosocomial spread especially in infants.

Several studies have been documented to have shown yearly shifts in relative predominance between group A and B strains (Hall *et al.,* 1990; Waris 1991; Peret*et al*, 1998). However, an epidemiologic pattern and any immunologic and clinical implications may be difficult to detect as the isolates examined in anyone site were generally few in number and were mainly from hospitalized individuals. Never the less there was a clear group shift demonstrated in this study with group A

predominating in 2009 to early 2010 and group B predominating in late 2010-2011. Peret*et al* (1998) observed that there is no genotype or subtype that predominates for more than one season and hypothesized that this affects the protective immunity in the population (Peret*et al.*, 1998). Lack of protective immunity in the community enables the virus to transmit more effectively.

All RSV A sequences were genetically related and the same was seen with the RSV B group but some isolates were 100% identical. In describing the molecular epidemiology of RSV, Cane and Pringle showed more of temporal relatedness than geographical relatedness, they showed isolates from widely distant parts clustering together which included isolates from UK, USA, Australia and Finland and close clustering was between isolates collected at the same time. (Cane & Pringles, 1995). Generally, similar groups were isolated in the same disease season, indicating a clear temporal relationship. This scenario supports the concept that some virus strains can be transmitted over broad geographic regions during a single epidemic season. However, the appearance of identical sequences does not necessarily prove that transmission of HRSV strains occurs between communities. (Peret*et al* 2000)

RSV spread in the hospital wards can be from patient to patient, patient to healthcare worker or healthcare worker to patient. Patients in the pediatric wards require frequent hands on care, which is provided by multiple healthcare providers and guardians therefore increasing their potential to community pathogens (Heerens *et al*, 2002).

The spread can be through small particles aerosols, (10µm mass media diameter) usually generated by coughing and sneezing, by large particle droplets which requires close person to person contact and by self-inoculation after touching contaminated surfaces (Hall, 2000). Most developing countries face challenges in controllingHAIs. Kenyan hospitals lack dedicated resources and administrative support for infection control activities in hospitals, and many hospitals face challenges such as inadequate facilities and lack of enough personnel. In addition, high patient volumes and short staffing can overwhelm even the most dedicated healthcare professionals. KNH, NNPGH and MDH are hospitals which are congested in nature with strained facilities and personnel. Consequently, there is no proper

ventilation in the wards, sharing of facilities like beds in general wards and there is also likelihood of sharing equipment like ventilators in the facilities. Infection control measures might be hard to be adhered to due to poor staffing, for example one healthcare worker might be expected to attend to so many patients therefore ends up handling patients after patient without using protective gowns or even not changing gloves in between patient.

5.2 Conclusion and Recommendations

The findings of this study presents the first ever study on nosocomial transmission of RSV in the three referral hospitals in Kenya. This study suggests that there is a likelihood of nosocomial transmission of RSV within the hospitals and the virus significantly affects children under two years of age consequently the paediatric ward was the most affected. Both RSV A and RSV B were observed to be in circulation.

RSV infection control measures should be put in place to reduce its spread in the hospitals. This can be achieved by most importantly creating awareness of RSV characteristic and its nature of infection and strengthening the infection control units in the hospitals through continuous medical education for the staff. planning and implementing feasible and effective control measures has been documented to be an important step in controlling nosocomial RSV (Hall, 2000) and other respiratory infections. These measures include; Hand washing, use of proper protective equipment and infected patient isolation. Hands that are used to care treat and comfort plays an important role in the spread of infection, therefore proper hand washing with soap and clean water should be recommended. Plans for future vaccines should target children less than two years old who were found to be the most affected.

Phylogenetic analysis revealed more of temporal relationship than geographical relationship between the isolates, this is likely to suggest that the virus can be widely spread during an epidemic. Additional study on full length genome should be done to see if there are changes elsewhere in the genome. This will help explain the presence of healthcare transmission of RSV and also enlighten on the groups to be considered in vaccine development

5.3 Study Limitations

Cases of health-care associated RSV may have been missed because of fever requirements as a selection criterion, not all RSV cases present with fever especially in very young children.   We may have classified some cases as health- care associated when actually the infection was acquired from the community before admission. The study protocol was limited to collecting samples from the patients alone, not healthcare workers yet they are also potential sources of spread.

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APPENDICES

APENDIX I: RNA EXTRACTION

1. Abbreviations

   1.1. AVE   Elution Buffer
   1.2. AVL   Lysis Buffer
   1.3. AW1   Wash Buffer 1
   1.4. AW2   Wash Buffer 2
   1.5. BSL   Biosafety Level 2 or 3
   1.6. NP   Nasopharyngeal
   1.7. OP   Oropharyngeal
   1.8. PCR   Polymerase Chain Reaction
   1.9. PPE   Personal Protective Equipment
   1.10. RNA   Ribonucleic acid
   1.11. rpm   Revolutions per minute
   1.12. RT-PCR   Reverse Transcriptase PCR
   1.13. RT PCR   Real Time PCR

2. Procedure

   2.1.1. Vortex the NP/OP sample and then add 100 µl of the sample to the
           aliquot of buffer AVL-carrier RNA solution. If the sample volume to be
           used is larger than 100µl, the buffer AVL-carrier RNA solution should
           be increased proportionately (in the ratio 1:4).

   2.1.2. Mix thoroughly by vortexing for 15 seconds so as to ensure efficient
           lysis.

   2.1.3. Incubate at room temperature for 10 minutes to allow for complete
           lysis of the virus.

   2.1.4. Add 400ul of ethanol (96-100%) to each sample and mix thoroughly
           by vortexing for 15 seconds. If the sample volume used is larger than

100μl, the amount of ethanol to be added should be increased proportionately (in the ratio 1:4).

2.1.5. Transfer 630μl of each sample from the previous step into the column supplied in the kit.

2.1.6. Centrifuge the column for 1 minute at 8000 rpm at room temperature and discard the filtrate.

2.1.7. Place the column in a clean 2ml collection tube.

2.1.8. Transfer the rest of the sample into the column, (**if the sample volume was greater than 100μl, repeat this step until all of the lysate has been loaded into the spin column**).

2.1.9. Centrifuge the column for 1 minute at 8000 rpm at room temperature and discard the filtrate.

2.1.10. Place the column in a clean 2ml collection tube.

2.1.11. Add 500μl of AW1 buffer to each column, it is not necessary to increase the volume of buffer AW1 even if the original sample volume was larger than 100μl.

2.1.12. Centrifuge the column for 1 minute at 8000 rpm at room temperature and discard the filtrate.

2.1.13. Place the column in a clean 2ml collection tube.

2.1.14. Add 500μl of AW2 buffer to the columns.

2.1.15. Centrifuge for 3 minutes at 14,000 rpm at room temperature and discard the filtrate.

2.1.16. Place the column in a clean 2ml collection tube.

2.1.17. Centrifuge for 1 minute at 14,000 rpm, (**this step is recommended in order to remove any residual buffer AW2 in the eluate that may interfere with downstream applications**).

2.1.18. Place the column in a clean 1.5ml tube; discard the old collection tube containing filtrate.

2.1.19. Add 100μl AVE buffer equilibrated to room temperature to columns and incubate for 10 minutes at room temperature.

2.1.20. Centrifuge for 1 minute at 10,000 rpm at room temperature.

2.1.21. Place the RNA on ice if PCR is to be carried out on the same day. However for short term storage, the viral RNA should be stored at -200C and -800C for long term storage. **(Viral RNA is stable for up to one year when stored at -20^0C or -80^0C)**.

APENDIX II: PCR AMPLIFICATION

Prepare reaction mix as below for 1sample

1st Step PCR

reagent	volume
Nuclease free water	11.3μl
5x buffer	5.0μl
dNTPS	1.0μl
AG20Forwardprimer	0.5μl
F164 reverse primer	0.5μl
RNase Out	0.2μl
Enzyme mix	1.0μl

Dispense 20 ul of the master mix into a 96 well plate

Add 5ul of RNA template and seal using a sealer and take to the thermo cycler.

1st step thermo cycling conditions.

30 min at 50^oC,15 minute at 95^oC and then 40 cycles of 1 minute at 95 ^{o}C, 1 minute at 55 ^{o}C and 1 minute at 72 ^{o}C.

2nd stepPCR

Prepare reaction mix as below for 1sample

reagent	volume
Nuclease free water	21μl
25μM MgCl$_2$	1.0μl
TaqManMastermix	25.0μl
BG10 Forward primer	0.5μl
F1 reverse primer	0.5μl

dispense 48ul of your master mix reagent in a 96 well plate then

Add 2 ul of your 1round pcr products, then take them to the thermo cycler

2nd Step thermocyclic conditions

2 minutes at 95°C, followed by 40 cycles for 1 minute at 95°C, 1 minute at 50 ^{o}C and 2 minute at 72°C.

APENDIX III: GEL ELECTROPHORESIS

3. Preparing Agarose gels

3.1.1. To prepare 2% Agarose gel, weigh 4 of Agarose, put the Agarose in a flat bottomed conical flask and add 200ml of 1X TAE electrophoresis buffer.

3.1.2. Dissolve the Agarose in the 1X TAE electrophoresis buffer by heating in a microwave oven with occasional mixing until no granules of Agarose is visible. Observe clear solution.

3.1.3. Weigh the flask and adjust to the original weight with deionized water to compensate for evaporation.

3.1.4. Cool the solution to about 60ºC with running water. Wear a N95 mask to **avoid inhalation of vapour!**

3.1.5. Add 5ul Ethidium bromide to the gel to facilitate visualization of DNA after electrophoresis.

3.1.6. Prepare the casting tray and insert combs. Pour the gel into a casting tray containing sample combs and allow solidifying at room temperature.

3.1.7. After the gel has solidified, pour a little TAE buffer and then remove the comb carefully so as not to rip the bottom of the wells. Orient the gel so that the wells are on the cathode side of the tank.

3.1.8. Add enough 1X TAE electrophoresis buffer to fill reservoirs and overflow the surface of the gel to a depth of 2-3 mm.

3.1.9. Prepare sample or loading as described below and load the samples into the sample wells formed by the comb.

3.2. **Preparing samples for loading the gel**

    3.2.1. DNA will migrate towards the anode that is usually colored red. The wells therefore should be placed towards the cathode side.

    3.2.2. Mix 3ul of loading dye (Bromophenol blue) with 1.5ul of the DNA ladder (marker) and 8ul of deionized water.

    3.2.3. Pipette 10ul of this into the first well.

    3.2.4. Mix 10ul of each sample DNA with 2ul of the loading dye and load 1 sample/well and note the positions for each sample in your laboratory note book.

Place the safety cover on the unit and connect the power leads on the apparatus and apply 100mV current. Confirm that current is flowing, in the expected direction, by observing the bubbles coming off the electrodes and ensuring proper connection of cathode and a

3.3. **Gel Photography**

    3.3.1. When adequate migration has occurred (approximately after 1 hour and at least ¾ the length of the gel).Transfer the gel to a UV transilluminator for visualization of the DNA fragments. Ethidium bromide intercalates between bases of DNA and gives an orange colour under UV light.

    3.3.2. Place the stained gel directly on top of a 300nm or 254 nm transilluminator.

    3.3.3. Wear an appropriate face-shield to shield your eyes from UV exposure and take photo of the gel. Ensure minimal exposure to the UV light.

        Record the name of sample as loaded

APENDIX IV: CYCLE SEQUENCING PCR

NB: The concentration of the primers used should be 1 picomole per μl.

Prepare 2 reaction mixes, 1 for the forward primer and another for the reverse primer.

reagent	volume
Big dye	1μl
Buffer	1μl
Primer	1.6μl
Nuclease free water	5.4μl
Purified PCR product	1μl

Put the reaction plate into the thermocycler and run with the following parameters

Denaturation 95°C

Hybridization between 50-55μElongation 60°C

CYCLE SEQUENCING PCR CLEAN-UP

NB: Freshly prepared 70
% Ethanol is used for the washing step

1. Into the PCR product add 1μl EDTA,1μl Sodium acetate (3M pH 5.2), 25μl Absolute Ethanol, seal and vortex.

2. Spin at maximum for 30mins, drain off the liquid.

3. Wash by adding 100μl of 70% Ethanol.

4. Spin at maximum for 10mins

5. Drain the Ethanol and dry off the plate (uncovered) at 60°C for 5 minutes in the thermocycle

6. Add 10μl HIDI

7. Denature at 95°C for 3mins

8. Place the plate in the sequencing machine for sequencing to take place.