
Running title: Respiratory Syncytial Virus, molecular characterization, referral Hospitals

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Summary

Background: Respiratory syncytial virus (RSV) is a major cause of community acquired severe respiratory illness in infants, immunocompromised individuals and the elderly. Limited information exists on the contribution of RSV in respiratory Hospital Associated Infection (rHAI) in developing countries.

Objective: To characterize Respiratory Syncytial Virus in the three Kenyan referral setting as a potential contributor to respiratory hospital acquired infection.

Methods: The study targeted all patients whose samples tested positive for RSV from the ongoing surveillance on healthcare associated respiratory infections. The study collected nasal and oropharyngeal samples from patients who developed new–onset axilla fever and influenza like illness, in patients who had been afebrile for at least three (3) days in the wards and tested them for different respiratory pathogens (Influenza A and B, Parainfluenza, Human metapneumovirus and adenovirus) alongside RSV. During this period A total of 37 samples tested RSV positive. These were characterized as RSV–A and –B using RT–PCR. Those that typed successfully were then sequenced in the attachment G protein and phylogenetically analyzed.

Results: Of the 37 samples, 13(35%) were RSV A, 6 (16%) RSV B, 1 (3%) was AB and 17 (46%) did not type. Twenty out of the 37 attained the sequencing criteria and only seventeen gave successful sequences. Three RSV– A and 2 RSV–B sequenced samples from KNH were 100% identical in the G ectodomain sequences. One RSV–A specimen from MDH and one RSV–A positive from NNPGH had 100% identity. Three sequences from KNH clustered with high nucleotide sequence identity. Children below 2years were significantly more at risk of RSV than those aged 5years and above (aOR=0.21, p=0.012).

Conclusions: The study inferred possibility of spread of RSV within the hospitals especially the paediatric ward. Any interventions to curb the spread should specifically target all children ≤ 2 years.

Introduction
Hospital associated infections substantially contribute to morbidity and mortality, represent an important public health concern and also increases both direct and indirect costs of patients’ care[1]. 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 [2]. Low resource setting fosters nosocomial spread of infection due to overcrowding, multiple bed occupancy as well as absence of space for cohorting and quarantine.

Viruses are important causes of lower respiratory tract infection [LRTI] in developing countries [3] and RSV is one of the most common causes of viral LRTI [4]. Human respiratory syncytial virus [HRSV] is the leading cause of severe lower respiratory tract infection in infants and young children[5]. The virus is the most common cause of nosocomial infection in paediatric wards with up to 45% of contacts acquiring infections [6]. It presents with 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 causes a cold and bronchial cough, while in infants and toddlers it causes bronchiolitis or pneumonia. RSV also causes serious pulmonary disease and death in adults particularly the elderly[7] and the immunocompromised individuals [8].

In a prospective surveillance conducted between 2002 and 2007 in Kilifi district, Kenya, the prevalence of RSV was documented to be 27% with a 2.2% mortality rate, with more than 1000 hospitalizations per 100,000 infants [9].

This study sought to characterize nosocomial RSV transmitted within three referral hospitals by sequencing and to determine the age distribution of the patients with the virus.

Methods
Study sites and population
This was a lab based descriptive study that was nested within an ongoing hospital associated infection surveillance study in Kenya conducted by KEMRI/CGHR under the Infection Control Programme [10]. The study used archived nasopharyngeal and oropharyngeal samples collected from 1st September 2009 through 31st August 2011. [Protocol number 2571]

The samples were collected from Kenyatta National Hospital [KNH], New Nyanza provincial general [NNPGH] and Mbagathi District Hospital [MDH]. The hospitals were selected on the basis of their geographical and public status, i.e. district, provincial or referral hospital status. KNH 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 wards and the special units NNPGH is a provincial referral hospital located in Kisumu. It is the largest public hospital in Nyanza province Kenya. It receives referrals from the western part of Kenya. It has 300 beds, 18,300 admissions and 13,306 outpatient visits annually.

MDH is located in Nairobi. The hospital serves the general population in Nairobi and is among the largest TB hospital in Nairobi therefore receiving a vast number
of pneumonia cases; it is also classified as an infectious
disease hospital. It has 200 beds with annual
admissions of 12900.

The study population consisted of all patients who had
been admitted in the specific wards of study, the
paediatrics wards, general wards, intensive care unit
and the special units.

Sample Size calculation
The population of interest are the RSV positive cases
collected between September 2009 to July 2011, these
were 40 in number; thus a finite population.
Thus for the sample size we used the below formula

\[ n = \frac{p(1-p)Z^2}{e^2} \]

\( p = 0.50 \)
\( Z_{\alpha/2} = 1.96 \) at \( \alpha = 0.05 \) and 95 % level of confidence
\( e = 0.05 \)
Thus giving \( n = 384.16 \)
Recalling that the population of the RSV Positive cases
has a finite number we employ a sample size
adjustment factor, i.e. the finite population correction
factor.
\[ n_{corrected} = n \times \frac{N}{N+n} \]

Where \( n \) is the uncorrected sample size, and \( N \) is the
population size.

In our case
\( n = 384.16 \)
\( N = 40 \)

Hence the corrected sample size for the laboratory staff
sample is
\[ n_{corrected} = \frac{384.16 \times 40}{40 + 384.16} \]
\[ n_{corrected} = 36.2278 \]
Thus approximately the corrected sample size for the
laboratory sample is 37

Sample collection
The study targeted all the patients whose specimen
tested positive for RSV from the ongoing study for the
healthcare associated infections and used archived
samples [Protocol number 2571] where Nasal and
oropharyngeal specimen were collected from patients
who developed new–onset axilla fever, in patients who
had been afebrile for at least three [3] days in the
wards. Details of the study are described elsewhere
[10]. Based on clinical information collected on
suspected HAI cases, suspected HAI RSV was defined
as a patient with new–onset fever/hypothermia with
concurrent respiratory signs and symptoms which were
not present at the time of admission. Since the
incubation period for RSV is 4 to 6 days, samples from
patients who tested positive for RSV and were in the
ward for less than 7 days were excluded from this
analysis.
Screening of the samples was done using real time
Reverse Transcriptase PCR [rt–RT PCR] to identify all
the RSV positive samples alongside other viruses[10]
[Protocol number 2571]. Group specific PCR was
carried out to classify the RSV positive samples into
Extracted viral RNAs were reverse transcribed and
amplified in a one-step reaction protocol [QIAGEN, Ltd]
with primers targeting the RSV G gene [AG20 and F164][12]. For the second round PCR A microlitre of the resultant products was further amplified in nested PCR procedure with the primers BG10 and F1, as previously described [12]. The product was visualized by ethidium bromide staining gel electrophoresis on a 2% agarose gel to confirm the success of amplification and the G gene PCR product was purified using GFX purification kit [GE, Healthcare, UK Limited ].

Four primers [5pmol/ul] were used in the RSV G–gene BigDye sequencing reaction on a 3130xl sequencher [Applied Biosystems]. Contig assembly was done in sequencher 5.10 [Gene codes corporation, USA ] [12] and the sequence alignments were done in BioEdit. Phylogenetic trees were generated in MEGA 5 program [13] and trees constructed using maximum likelihood method. The appropriate model of evolution was determined in jmodeltest. 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.

Data Analysis
Data was analyzed using STATA [version 11; Stata Corporation, College Station, TX]. Descriptive Statistics was used to describe distribution of patients by their demographic characteristics, by gender of the patients, the hospitals to which they were admitted, age distribution [which was categorized into those under 2 years, between 2 to 5 years and those above 5 years] and also distribution by ward type. The wards included the paediatrics unit, intensive care unit, burns unit, surgical wards and the medical ward.

Ethical Clearance
This study was approved by the KEMRI Scientific Steering Committee and the KEMRI National Ethical Review Committee [protocol SSC No. 2571]. Consent to store aliquots of the samples for further analysis was sought from the participants prior to sample collection and the minors were assented for according to the World Health Organization guidelines.

Results
Demographics and clinical characteristics
From September 2009 to August 2011 a total of 37 patient specimens [15%] tested positive for RSV by routine real time PCR viral screening. Of these, 13[35%] were RSV A, 6 [16%] RSV B, 1 [3%] AB and 17[46%] did not type. Of the twenty [54%] samples that were sequenced, 17 [85%] were successfully sequenced [9 KNH, 4 MDH and 4 NNPGH]. Of the sequenced specimen, 56% were from males with majority of the patients [50%] being from Kenyatta National Hospital. Children below 2years were significantly more at risk of RSV than those aged 5 years and above [aOR=0.21,p=0.012]. There was no statistically significant difference in the risk for children 2–4yrs compared to children below 2years [aOR=0.85,p=0.7342].

Phylogenetic analysis
Phylogenetic tree analysis of the RSV A sequences [figure 1] showed that all the samples collected from the hospitals were genetically similar and clustered together
as one clade. Three RSV group A sequenced samples from KNH [39/09, 40/09 and 41/09] were identical in the G ectodomain sequences with 63% bootstrap value and near 100% nucleotide similarity. Two samples, both RSV-A specimens [MDH, 023/2010, NNPGH, 021/2010], were over 90% identical although these hospitals are approximately 160 miles apart. All the sequences in group B belonged to one genotype, the BA1 genotype, but they were in 3 different clusters which were not hospital specific. The sequences had genetic differences within the clusters but two samples from KNH [176 and 177] had almost 100% nucleotide similarity with a bootstrap value of over 90%. The sequences collected from the three referral hospitals were observed to be closely related despite their distances apart.

**Figure 1: Phylogenetic trees**
Figure 1: Phylogenetic analysis of RSV-A depicting virus genotypes identified during the study period. Closed circle show viruses detected from Kenyatta National Hospital, closed rectangle are samples from New Nyanza Provincial Hospital and closed triangle from Mbagathi District Hospital. The sample naming includes sample name–month of collection–year of collection.

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 they had both stayed in hospital for more than two months. It was also observed that all the patients except one HAINNPGH 019 had been in hospital more than two weeks before showing symptoms of RSV [Table 2 showing length of hospital stay before onset of RSV]
Figure 2: RSV–B phy. RSV–B phylogenetics showing genotypic relationship of viruses identified between the years 2009–2011. Closed circle show viruses detected from Kenyatta National Hospital, closed rectangle are samples from New Nyanza Provincial Hospital and closed triangle from Mbagathi District Hospital. The sample naming includes sample name–month of collection–year of collection.

Figure 3: Patient time of admission

Figure 3: Graph showing patient time of admission, date RSV A virus was detected and the outcome date.

Key

--- Duration of hospital stay before virus was detected

— Duration of hospital stay after virus detection

* Date of virus detection

• Date of outcome
### Table 1a: Demographic characteristics of all RSV positive samples

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Subtypes</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gender</strong></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>22 [59.5]</td>
</tr>
<tr>
<td>Female</td>
<td>15 [40.5]</td>
</tr>
<tr>
<td><strong>Age Group</strong></td>
<td></td>
</tr>
<tr>
<td>Adult</td>
<td>5 [13.5]</td>
</tr>
<tr>
<td>Child</td>
<td>32 [86.5]</td>
</tr>
<tr>
<td><strong>Hospital</strong></td>
<td></td>
</tr>
<tr>
<td>KNH</td>
<td>22 [59.5]</td>
</tr>
<tr>
<td>MDH</td>
<td>9 [24.3]</td>
</tr>
<tr>
<td>NNPGH</td>
<td>6 [16.2]</td>
</tr>
<tr>
<td><strong>Ward Type</strong></td>
<td></td>
</tr>
<tr>
<td>Burns Unit</td>
<td>1 [2.7]</td>
</tr>
<tr>
<td>ICU</td>
<td>4 [10.8]</td>
</tr>
<tr>
<td>Medical</td>
<td>1 [2.7]</td>
</tr>
<tr>
<td>Paediatrics</td>
<td>30 [81.1]</td>
</tr>
<tr>
<td>Special</td>
<td>1 [2.7]</td>
</tr>
<tr>
<td>Surgical</td>
<td>0 [0.0]</td>
</tr>
</tbody>
</table>

### Table 1b: Demographic characteristics and RSV samples that were sequenced

<table>
<thead>
<tr>
<th>Group</th>
<th>Characteristic</th>
<th>[N*=16] n [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gender</strong></td>
<td>Female</td>
<td>7 [43.8]</td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>9 [56.2]</td>
</tr>
<tr>
<td><strong>Hospital</strong></td>
<td>KNH</td>
<td>8 [50.0]</td>
</tr>
<tr>
<td></td>
<td>MDH</td>
<td>4 [25.0]</td>
</tr>
<tr>
<td></td>
<td>NNPGH</td>
<td>4 [25.0]</td>
</tr>
<tr>
<td><strong>Age Categories</strong></td>
<td>&lt;2 Years</td>
<td>12 [75.0]</td>
</tr>
<tr>
<td></td>
<td>2 to &lt;5 Years</td>
<td>4 [25.0]</td>
</tr>
<tr>
<td></td>
<td>5 Years and above</td>
<td>0 [0.0]</td>
</tr>
</tbody>
</table>

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Table 2: Multivariate analysis of demographic characteristics in relation to RSV positivity

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>RSV Positivity</th>
<th>Adjusted</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n/N [% Positive]</td>
<td>Adjusted Odds Ratio [OR]</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;2 Years</td>
<td>24/85 [28.2 %]</td>
<td>Reference</td>
<td></td>
</tr>
<tr>
<td>2~4 Years</td>
<td>7/36 [19.4 %]</td>
<td>0.85[0.33–2.17]</td>
<td>0.7342</td>
</tr>
<tr>
<td>5+years</td>
<td>6/127 [4.7 %]</td>
<td>0.21[0.06–0.71]</td>
<td>0.0117</td>
</tr>
<tr>
<td>Ward Type</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Paediatrics</td>
<td>1/5 [20.0 %]</td>
<td>Reference</td>
<td></td>
</tr>
<tr>
<td>ICU</td>
<td>4/59 [6.8 %]</td>
<td>0.63[0.17–2.39]</td>
<td>0.5002</td>
</tr>
<tr>
<td>Others</td>
<td>1/30 [3.3 %]</td>
<td>0.37[0.09–1.46]</td>
<td>0.1546</td>
</tr>
</tbody>
</table>

**DISCUSSION**

This study describes a retrospective investigation on respiratory samples collected from patients admitted to three referral hospitals in Kenya and were subsequently infected with RSV during their hospital stay. The study identified both group A and B genotypes were circulating in the three hospitals with limited diversity within the groups. Several studies have been documented to have shown yearly shifts in relative predominance between group A and B strains [14, 15]; However, in our study an epidemiologic pattern and any immunologic and clinical implications may be difficult to detect as the isolates examined in any one site were generally few in number and were mainly from hospitalized individuals. Never the less there was a clear group shift inferred in this study with group A predominating in 2009 to early 2010 and group B predominating in late 2010–2011. This mirrors Peret et al’s observation, that there is no genotype or subtype that predominates for more than one season and hypothesized that this drives changes in protective immunity in the population [15]. A lack of protective immunity in the community enables the virus to transmit more effectively. Identifying differences between strains within groups was achieved by phylogenetic analysis and all sequences in the different groups were observed to be genetically related. Isolation of viruses with identical sequences indicates a common source [15]. There were slight genetic differences within the clades but three group A sequences from Kenyatta National Hospital, KNH 039, KNH 040 and KNH 041 [Gene Bank accession numbers KJ556906, KJ556907 and KJ556908] and two group B sequences from the same hospital, KNH 176 and KNH 177 [Gene Bank accession numbers KJ556913 AND KJ556914] were above 90% identical.
This infers a possibility of a common source of infection within the hospital.

Incomplete immunity to RSV results in 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 [14] facilitates its nosocomial spread.

Patients in the paediatric wards require frequent hands on care which is provided by multiple healthcare providers and guardians therefore increasing their potential to community pathogens [16]. Most developing countries face challenges in controlling HAIs due to their congested nature. This may be the consequence of inadequate 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 inadequate staffing, of infection control personnel and lack of adequate infection control supplies.

The Centers for Disease Control [CDC] and WHO recommends frequent hand washing with soap and water, cleaning contaminated surfaces with disinfectants and reduced interaction between individuals with flu like symptoms with high risk children. As much as possible, limit the time high risk children spend in child–care centers or other contagious centers. The findings of this study suggest that there is a likelihood of nosocomial spread of RSV within the three hospitals. However, finding of the similar sequence between patients does not give the conclusion that there is nosocomial transmission therefore additional study on full length genome sequencing should be done. This will explain if there are changes elsewhere in the genome of the isolates.

Education on the transmission and control measures needs to be emphasized among the healthcare providers in order to intervene in the nosocomial spread of RSV. This can be achieved by most importantly creating awareness of RSV characteristic and its nature of infection. Introducing education programs in the hospitals is an important key in infection control and this can be done through regular continuous medical education for the medical staff. Strict infection control measures should be put in place, planning and implementing feasible and effective control measures has been documented to be an important step in controlling nosocomial RSV [14] and other respiratory infections. These measures include; Hand washing, use of proper protective equipment and infected patient isolation. Any interventions to curb the spread should majorly target children under 2 years of age.

The main limitation in this study was the fact that it was not possible to determine source of HA–RSV [health care worker, other patient, or visitor]. Having temperature of more than 38 °C as a major criteria of inclusion to the study could have possibly limited the chances of capturing asymptomatic cases of RSV, since RSV does not necessarily present with fever.
References


12. Agoti CN, Mwihuri AG, Sande CJ, Onyango CO, Medley GF, Cane PA, et al. Genetic relatedness of infecting and reinfecting respiratory syncytial virus strains identified in a birth cohort from rural

