TITLE: Respiratory virus-associated severe acute respiratory illness (SARI) and viral clustering in Malawian children in a setting with a high prevalence of HIV, malaria and malnutrition

Running title: Paediatric SARI and viral clustering Malawi

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Abstract (word count=200)

Background: We used four years of paediatric severe acute respiratory illness (SARI) sentinel surveillance in Blantyre, Malawi to identify factors associated with clinical severity and co-viral clustering.

Methods: From January 2011 to December 2014, 2363 children aged 3 months to 14 years presenting to hospital with SARI were enrolled. Nasopharyngeal aspirates were tested for influenza and other respiratory viruses. We assessed risk factors for clinical severity and conducted clustering analysis to identify viral clusters in children with co-viral detection.

Results: Hospital-attended influenza-positive SARI incidence was 2.0 cases per 10,000 children annually; it was highest children aged under 1 year (6.3 cases per 10,000), and HIV-infected children aged 5 to 9 years (6.0 cases per 10,000). 605 (26.8%) SARI cases had warning signs, which were positively associated with HIV infection (adjusted risk ratio [aRR]: 2.4, 95% CI: 1.4, 3.9), RSV infection (aRR: 1.9, 95% CI: 1.3, 3.0) and rainy season (aRR: 2.4, 95% CI: 1.6, 3.8). We identified six co-viral clusters; one cluster was associated with SARI with warning signs.

Conclusions: Influenza vaccination may benefit young children and HIV infected children in this setting. Viral clustering may be associated with SARI severity; its assessment should be included in routine SARI surveillance.

Background

It is estimated that worldwide, the case-fatality of severe pneumonia in children <5 years is 8·9%, which in 2011 amounted to 1.26 million deaths[1]. Much of this burden falls on sub-Saharan Africa where severe acute respiratory infection (SARI), including pneumonia, is a leading cause of childhood hospital attendance and death [2]. Although laboratory diagnostic facilities are rarely available in such settings, sentinel surveillance using multiplex molecular diagnostics has recently provided considerable insight into the true burden of disease and the complexity of SARI aetiology. Respiratory syncytial virus (RSV), parainfluenza viruses, rhinoviruses, influenza viruses and adenovirus have been commonly detected in SARI surveillance across the African continent [3-8]. While there are a few viruses where detection in respiratory disease cases is likely causal (e.g., influenza, RSV)[9, 10], for other commonly identified viruses causality has been difficult to determine. Use of multiplex assays has led to an increasing realisation that children with SARI commonly carry multiple viral pathogens which may potentially contribute to disease.

In the context of a low-income population with multiple drivers of immune compromise (e.g., HIV, malnutrition, malaria) [11], we conducted active surveillance at a large urban teaching hospital in Malawi to estimate the incidence of childhood SARI and explore the association of SARI clinical severity with HIV and clustering of respiratory viral co-infection. While previous studies have focused on children aged <5 years, we included children aged 3 months to 14 years in our analysis, to better capture the total burden and identify age groups particularly at-risk.

Methods

Study site, population and study design

QECH is the only government inpatient facility for Blantyre (population ~500,000 children aged <15 years); it offers care free at the point of delivery. Overall, 13% of children aged <5 years in Malawi are moderately to severely underweight and 4% are wasted; 80.9% of children aged 12-23 months have received all Expanded Program on Immunization vaccinations [12]. There is no national routine influenza vaccination in Malawi. In 2010, an influenza A(H1N1)pdm09 monovalent vaccine campaign achieved 74% coverage in pregnant women, and 7% of the overall population [13]. An estimated 2.5% of children aged <15 years are HIV infected [14]; HIV prevalence in children < 5 years on QECH non-surgical paediatric wards is estimated at 6%. Blantyre has two distinct weather seasons, a rainy season (January to April) and a cool dry season (May to August). Overall 25.2% of Paediatric Accident and Emergency Unit (PAEU) patients have a positive malaria blood slide; malaria presentations to the PAEU peak from December to May.

Patients aged 3 months to 14 years presenting during surveillance hours (weekdays, 8 am – 1 pm) from January 2011 through December 2014 were screened. Consecutive patients fulfilling the SARI case definition were recruited (maximum 5 per day). Demographic and clinical data were captured through an electronic data collection system [15]. Nasopharyngeal aspirates (NPA) were obtained and tested for influenza viruses; from 2011 to 2013 NPAs were also tested by multiplex assay for respiratory pathogens. Thick blood films for malaria were performed on all children.

SARI was defined as (a) an acute illness with symptom onset <7 days and (b) reported or recorded fever $\geq 38^{o}$ C (or hypothermia in children < 6 months). Additional criteria for SARI varied by age. In children < 6 months, additional criteria were: (c) cough or apnea - or - (d) any respiratory symptom requiring hospitalization. In children 6 to 59 months, an additional criterion was: (c) clinician diagnosed lower respiratory infection. In children 6 to 14 years, additional criteria were: (c) cough - or - sore throat and (d) shortness of breath - or - difficulty breathing. SARI with warning signs was considered clinically more severe and defined as admission to hospital - or - chest recession - or - blood oxygen saturation of ≤90%. In this resource-limited setting, some patients with severe illness requiring admission were sent home. Thus, hospital-attendance (not admission) was required for study enrolment.

Laboratory procedures

NPAs were stored at -80°C in Universal Transport Medium (Copan, Brescia, Italy)[16] and batch-tested for influenza viruses by real-time reverse transcription polymerase chain reaction (rRT-PCR). Total nucleic acids were extracted from 300µl aliquots of each specimen with the Qiagen BioRobot® Universal System using the QIAamp One-For-All nucleic acid kit (Qiagen ltd., Manchester, UK). The quantity of nucleic acid used per reaction was 5 µl for the CDC Human Influenza rRT-PCR diagnostic panel (CDC Influenza Division) detecting influenza A and B viruses and influenza A subtypes H1, H3, 2009H1 and H5N1 and 10 µl for the FTD respiratory pathogens 33 kit (Fast-track Diagnostics Ltd., Luxembourg). Details on sample processing with by FTD rRT-PCR are provided in Appendix 1. HIV serostatus was assessed by rapid test (Alere Determine™ HIV-1/2 and Trinity Biotech Uni-Gold™ HIV) according to WHO guidelines[17]. PCR for detection of HIV RNA was performed in children aged 3-11 months with a positive HIV rapid test. HIV infection was defined as a positive HIV rapid test (in the absence of a negative HIV PCR); data was not collected on HIV exposure.

Ethics Approval

Ethics approval for this study was obtained from the Liverpool School of Tropical Medicine Research Ethics Committee (Approval #RETH000790), the University of Malawi College of Medicine Research Ethics Committee (COMREC, Approval #958) and CDC through a reliance on COMREC. Informed consent was obtained from guardians of all study participants.

Data analysis

Numerators for minimum SARI incidence estimates were generated by summing the number of cases resident in Blantyre within strata of age category and HIV status. Numerators were adjusted by multiplying by the reciprocal of the daily proportion of recruited cases among all SARI cases attending the PAEU. Denominators for HIV and age strata were derived by applying age-specific HIV prevalence estimates to census figures for Blantyre District's population aged 0-14 years [18]. The former were obtained by apportioning total HIV prevalence among Malawian children aged <15 years [14] according to the age-distribution of paediatric HIV from Mozambique which borders Malawi, and has a similarly severe HIV epidemic [19] [20]. Estimates of age-specific HIV prevalence were unavailable for Malawi for the study period. Incidence was obtained by dividing numerators by denominators and multiplying by 10,000; HIV-associated Incidence Rate Ratios (IRR_{HIV}) were calculated by dividing incidence in HIV infected strata by incidence in HIV un-infected strata; 95% confidence intervals (95% CIs) of incidence and IRR_{HIV} were generated with 1000 bootstrap samples.

Data analysis was performed using SAS® 9.3 (SAS Institute, Cary NC). Temporal trends in weekly sample counts for SARI cases were assessed by plotting 5-week moving averages of sample counts by recruitment week. We developed two logistic regression models with a binary outcome factor for the child's clinical status. The first outcome represented SARI with warning signs (i.e. clinical markers of very severe illness) vs. SARI without warning signs. The second outcome represented influenza positive SARI vs. influenza negative SARI. Auto-regressive correlation of residuals was removed by introducing a patient-level Kernel weighted moving average of the prior probability of case status. Parsimonious models were developed by stepwise logistic regression, retaining age, sex a priori, and explanatory factors with a 2-sided p-value of <0.05. Adjusted relative risk ratios for factors associated with the outcomes, were derived from these models.

Detection of multiple viruses in SARI is common, with many possible combinations of viral carriage. Conventional statistical techniques (eg. regression models, covariance matrices and temporal plots) have limited capacity to quantify, characterize or identify factors associated with viral carriage groupings. To assess multiple virus carriage clusters in our setting, we performed 'nearest-neighbour' discrete hierarchical cluster analysis in patients with viral co-detection using Gower's distance [21]. Distance was based on similarity of viral pathogens detected in the nasopharynx of SARI patients; each patient was a member of only one cluster. We defined clusters as those which increased the R-squared by $\geq$0.05 (using Ward's method); to improve precision, 10% of observations with the lowest densities were discarded. Using, univariate logistic regression we identified factors associated with cluster membership.

Results

SARI population

From 1 January 2011 to 31 December 2014, 2363 SARI cases (median age: 15 months, interquartile range [IQR]: 8 - 27 months) were recruited. In total, 605/2260 (26.8%) SARI cases had clinical warning signs (Table 1, Consort diagram –Appendix 2). Warning signs were determined as follows: 489/605 (80.8%) were hospitalised (median duration of stay 2 days [IQR: 1, 3]); 37/605 (6.1%) had blood oxygen saturation <90%; 75/605 (12.4%) had chest recession; 4/605 (<1%) had both of these clinical features. In cases aged 3 to <12 months, 17/247 (6.9%) had a positive HIV test result compared to 29/563 (5.2%) cases aged 12 to <36 months, 45/1050 (4.3%) cases aged 36 to 59 months, 19/241 (7.9%) cases aged 5 to 9 years and 18/103 (17.5%) cases aged 10 to 14 years. Eight of 17 HIV infections in cases aged 3 to <12 months (47.1%) were confirmed by PCR.

Viruses detected in association with SARI

We detected influenza viruses in 266/2363 (11.3%) SARI cases. When tested for the extended panel of pathogens, influenza viruses A and B (any type) were detected in 201/1835 (10.9%), rhinoviruses in 358/1835 (19.5%), RSV in 220/1835 (11.9%) and adenovirus in 162/1835 (8.8%). In 542/1835 (30%) SARI cases, no viral pathogen was detected (Table 2).

Seasonality of influenza and RSV

Plots of weekly influenza positive SARI cases suggest both unimodal and bimodal (2 peaks per year) seasonality. Weekly influenza-positive SARI cases increased during the rainy season (January to April) in all four years of surveillance. A second peak of influenza-positive SARI cases occurring in September to October was confined to 2013 and 2014 (Figure 1). In multivariable analysis, influenza detection in SARI increased in the rainy season (adjusted risk ratio [aRR]: 3.3, 95% CI: 1.9, 5.4) and the cool dry months (May to August) (aRR: 2.1, 95% CI: 1.2, 3.6), compared to September to December (Table 3). Influenza detection in SARI was significantly higher in the rainy season compared to the cool dry season (aRR: 1.6, 95% CI: 1.0, 2.5). The predominance of influenza virus types varied within and between years. Influenza A(H1N1)pdm09 was most prevalent in the first half of

2011 and 2013; influenza A(H3N2) and influenza B viruses were most prevalent in 2012, the latter half of 2013 and in 2014. In contrast, RSV infection displayed regular seasonality, with peaks in the first half of the rainy season (January to March) (Figure 1).

Incidence estimates for SARI and respiratory virus-associated SARI

SARI incidence was 17.5 cases per 10,000 children annually, with highest incidence in children aged 3 to 11 months (89.5, 95% CI: 85.8, 93.0). Influenza-positive SARI incidence was 2.0 cases per 10,000 children annually and was highest in children aged 3 to 11 months (6.3, 95% CI: 5.3, 7.6). Incidence of RSV positive SARI per 10,000 children annually was 4.6 (95% CI: 0.1, 15.8) and was highest in children 3 to 11 months (17.3, 95% CI: 13.7, 18.6) (Table 4).

Risk factors for SARI with warning signs and virus-associated SARI

We found 390/1505 (25.9%) SARI cases had warning signs, among whom 309/390 (79.2%) were hospitalised. In multivariable analysis, RSV was the only pathogen associated with SARI with warning signs (aRR: 1.9, 95% CI: 1.3, 3.0). Nevertheless, 52/249 (20.9%) influenza positive SARI cases required hospitalisation. A positive HIV test was associated with a 2-fold increased risk of SARI with warning signs (aRR: 2.4, 95% CI: 1.4, 3.9) (Table 5) as well as increased incidence of SARI, SARI with warning signs and influenza positive SARI (Table 4). HIV-associated incidence rate ratios (IRR_{hiv}) rose with increasing age. The $IRRs_{hiv}$ for SARI with warning signs was 2.6 in children 3-11 months compared to 37.7 in children 10-14 years. In children aged >5 years, incidence of hospital-attended influenza positive SARI was at least 8-fold higher in HIV infected children compared to the HIV uninfected. There was no difference in the incidence of RSV-positive SARI between HIV infected and HIV uninfected children.

In multivariable analysis, controlling for aetiology, SARI patients recruited during the rainy season (January-April) were more than twice as likely to have warning signs compared to patients enrolled during September-December (aRR: 2.4, 95% CI: 1.6, 3.8) (Table 5). Peaks in RSV and influenza activity corresponded to peaks in the occurrence of SARI with warning signs (Figure 1). Detection of RSV in cases of SARI warning signs was much higher during the rainy season (39.8%) compared to other times of year (5.9%).

The adjusted risk ratio for positive influenza test in SARI increased with older age and rainy season of recruitment (Table 3). After adjusting for age, gender and HIV status, rainy season recruitment was significantly associated with SARI with warning signs in influenza positive SARI patients (aRR:3.42, 95% CI: 1.37, 8,53 – analysis not shown). In adjusted analysis, influenza A (H1N1)pdm09 virus was associated with double the risk of SARI with warning signs, compared to other influenza sub-types (aRR:2.10, 95% CI: 0.98, 4.53 – analysis not shown).

Co-viral infection, viral clustering and clinical severity in SARI

Detection of two or more viral pathogens by multiplex PCR occurred in 362/1835 (19.7%) SARI cases. Viral co-detection was highest in SARI cases positive for coronaviruses 229(70.6%) and enteroviruses (79.7%). Viral co-detection was least common in SARI cases testing positive for influenza A(H1N1)pdm09 virus (27.3%), influenza A (H3N2) virus (29.0%) and RSV (29.5%) (Table 2).

Viral co-detection *per se* was not associated with warning signs in SARI (Table 5). We used discrete hierarchical cluster analysis based on similarity of viral pathogens detected by multiplex PCR assay in SARI cases to explore whether particular groupings of viruses were associated with warning signs, host or seasonal factors. We identified six clusters, which accounted for 48.3% of the total variation in viral pathogen test results in children with co-viral detection. Cluster size ranged from 23 to 96 members; smaller clusters had fewer viral pathogens and lower within-cluster heterogeneity. Clusters were distinguishable by the type of viral pathogens detected. For example, 80% of influenza A (H3N2) virus was found in Cluster A; >65% of bocavirus detected was found in Cluster E (Appendix 3).

Cluster membership was significantly associated with clinical and temporal factors (Figure 2). Among children with co-viral detection, membership in Cluster D (characterized by influenza A(H1N1)pdm09 virus, RSV, coronaviruses 43 and 63) was associated with nearly double the risk of SARI with warning signs (OR: 1.9, 95% CI: 1.2, 3.5- analysis not shown), compared to other clusters. In Cluster D, 47/70 (67%) of members had RSV or influenza A(H1N1)pdm09 virus infection (Appendix 3); 11.4% of members had RSV/ influenza A(H1N1)pdm09 virus co-infection, accounting for all such co-infections in SARI. Rainy season recruitment was significantly associated with Cluster D, while dry season recruitment was significantly associated with Cluster B (characterized by parainfluenza viruses 2 and 3). Clusters were also significantly associated with temporal peaks in viral pathogen activity. For example, 65% of Cluster A members were recruited during a peak in influenza A(H3N2) virus activity occurring from September to December in 2013 (Figures 1 and 2), compared to 13.3% of other children with co-viral detection. Cluster membership was not associated with host factors (age, gender, HIV status, underweight).

Discussion

Hospital-attended SARI was common in this urban sub-Saharan African setting, particularly in infants 3-11 months, in whom incidence was 91.7 cases per 10,000 children annually. Similar to studies from other settings, influenza viruses and RSV were important SARI-associated pathogens [5-8, 22, 23], with prevalence rates of 11% and 12%, respectively. As elsewhere, HIV infection increased risk of SARI and presence of warning signs in SARI cases [24-26]. Among older children, HIV greatly increased risk of influenza positive SARI, consistent with data from South Africa[25].

Viral co-infection occurred in almost 20% of SARI cases, highlighting its potential impact in the development or clinical worsening of SARI [27]. Although co-viral detection *per se*, was not associated with clinical severity or season, we found one viral cluster, characterized by a high proportion of RSV and influenza A(H1N1)pdm09 virus infection, which was significantly associated with clinical warning signs and rainy season recruitment. Cluster members co-infected with RSV and influenza A(H1N1)pdm09 virus had a higher rate of warning signs, but the number of co-infected individuals (within the cluster and the entire sample) was too small to formally test for interaction. It is unclear therefore whether clinical severity in this cluster resulted from biological interaction of pathogens, additive risks from each pathogen or other underlying factors. Clusters clearly mapped to peaks and troughs in individual pathogen activity. We suggest that this viral clustering, which was associated temporal dynamics of pathogen activity may have arisen from complex virus-virus and host-virus pathogen interactions.

Clinical severity in SARI demonstrated seasonal peaks, coinciding with rainy season peaks in RSV activity. RSV was detected in 40% of SARI cases with warning signs recruited during the rainy season compared to 6% recruited other times of year. Thus RSV may drive rainy season increases in clinical severity in paediatric SARI in our setting, consistent with studies elsewhere in sub-Saharan Africa [28, 29]. Nevertheless, rainy season remained independently associated with increased risk of warning signs in SARI in multivariable analysis controlling for RSV, HIV and other viral pathogens. Therefore, the observed rainy season excess of clinical severity in SARI is in part attributable to unmeasured factors. We speculate that these factors include other intervening illnesses and seasonal malnutrition (in Malawi the rainy season coincides with the post-harvest ‘lean season’[30]). However, we cannot exclude seasonal differences in healthcare utilisation.

We acknowledge that our study has limitations. We did not recruit children aged < 3 months, in whom SARI-related deaths are known to be elevated[31]. We were unable to determine the role of bacterial pathogens in SARI, as we lacked laboratory data and systematic radiological data to identify probable infection in the context of a very high prevalence of bacterial carriage. Our estimates of SARI incidence by HIV strata were based on Mozambican paediatric HIV prevalence rates as we lacked data from Malawi. Nevertheless, Malawi and Mozambique have similar rates of antenatal HIV prevalence[12, 32, 33], and have similarly high rates of HIV-infected pregnant women accessing antiretroviral treatment[34]. We did not assess the impact of HIV exposure on SARI risk in HIV uninfected children. HIV exposure was associated with higher SARI incidence and greater SARI severity in HIV uninfected South African children[35].

In conclusion, SARI is common in this high HIV prevalence setting, where influenza viruses, rhinoviruses and RSV were the most prevalent viruses detected. HIV greatly increased risk of influenza-associated SARI in children, therefore yearly influenza vaccination should be considered in routine paediatric HIV clinical care. Influenza vaccination in HIV infected children is safe, however has low efficacy (< 20%) and may only be immunogenic in older children and adolescents with virological suppression [36-38]. Viral co-infection was common with one co-viral cluster associated with clinical severity in SARI cases. In this context, there is considerable potential for the use of multiplex respiratory virus assays in tandem with cluster analysis to reveal multiple-pathogen associated outbreaks and disease burden. This approach may expose the potential for synergistic effects of vaccine strategies that disrupt viral clusters. Vaccine probe studies to assess the impact of viral co-infection on clinical severity, could clarify complex pathogen and host interrelationships and reveal the true burden of disease.

Disclaimer: The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention.

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Instructions for figures:

The three files 'PanelA_Influenza(All Types).png', 'Panel B_Influenza Type by week.png' and ' PanelC_RSV.png' form Figure 1. The title of figure 1 is 'Seasonal plots of SARI with warning signs, influenza and RSV in paediatric SARI cases, Blantyre, Malawi, 2011-2014'.

For 'PanelA_Influenza(All Types).png' the panel title is 'A. Influenza (All Types)'; for the legend please note that the red line is 'Influenza positive SARI', the dotted black line is SARI with warning signs and the dotted grey line is SARI cases tested.

For 'Panel B_Influenza Type by week.png' the panel title is 'B. Influenza Type by Week'; for the legend please note that that red bars are for 'A (H1N1)pdm09', green bars are for 'A (H2N3)', yellow bars are for 'B' and purple bars are for 'Other types'.

For 'PanelC_RSV.png' the panel title is 'C. RSV'; for the legend please note that the red line is 'RSV positive SARI', the dotted black line is SARI with warning signs and the dotted grey line is SARI cases tested.

The file 'Figure 2 Dendrogram of co-viral clusters.png' is for figure 2. The title of the figure is 'Figure 2. Dendrogram of co-viral clusters'. The notes with the title are: 'Six co-viral clusters (A-F) were identified in 362 paediatric SARI cases, in whom >2 viral pathogens were detected in the nasopharynx. Each SARI case is a member of only cluster; clusters membership is based on similarity of viral pathogens detected. As shown here, characteristics such as SARI severity, number of viruses detected per child, and particular season and year of recruitment are more common in some clusters than others.'

For Figure 2's legend, referring to the first line of coloured bars, green bars are 'SARI without warning signs' and red bars are 'SARI with warning signs'; referring to the second line of coloured bars, bluish-grey bars are 'Number of viruses detected <3' and orange bars are 'Number of viruses detected ≥3'; referring to the third line of coloured bars, lavendar bars are 'Recruited in rainy season' and yellow bars are 'Not recruited in rainy season'; referring to the fourth line of coloured bars, grey bars are 'recruited in 2011', blue bars are 'recruited in 2012', pink bars are 'recruited in 2013' and light green bars are 'recruited in 2014'.

For the file named 'Appendix 2 CONSORT Diagram.png' the title is, 'Appendix 2. CONSORT Diagram of data analyses'.

For the file named, 'Appendix 3.png' the title is, 'Appendix 3. Viral pathogens detected in six clusters identified by discrete hierarchical cluster analysis of paediatric SARI cases with co-viral detection'. For the legend, BocaV is 'Bocavirus', HMPV is 'Human metapneumo virus', Cor63 is 'Coronavirus 63', H1N1 is 'Influenza A(H1N1)pdm09', AV is ‘Adenovirus’, Para 3 is ‘Parainfluenza virus 1’, Cor43 is ‘Coronavirus 43’, RSV is ‘Respiratory syncytial virus’, PV is ‘Parechovirus’, Para 2 is ‘Parainfluenza virus 2’, FluC is ‘Influenza C’, Rhino is ‘Rhinovirus’, Cor229 is ‘Coronavirus 229’ and H3 is ‘Influenza A (H2N3)’.

Table 1. Paediatric severe acute respiratory illness (SARI) patient characteristics by clinical severity and hospitalisation status, Blantyre, Malawi, 2011-2014

	All N(%)[3]	SARI without warning signs[1] N(%)	SARI with warning signs N(%)	p-value[2]	Non-Hospitalised SARI N(%)	Hospitalised SARI N(%)	p-value[2]
Total	2260	1655	605		1771	489	
Female	1134 (43.0)	850 (51.4)	205 (33.9)	0.011	855 (48.3)	205 (41.9)	0.017
Age							
3 to <6 months	265 (11.7)	207 (12.6)	58 (9.6)		240 (12.8)	43 (8.8)	
6 to <12 months	584 (25.8)	423 (25.6)	161 (26.6)		483 (25.8)	129 (26.4)	
12 to <36 months	1077 (47.7)	777 (46.9)	300 (49.6)		862 (46.0)	244 (49.9)	
36 to <60 months	248 (10.9)	192 (11.6)	56 (9.3)		212 (11.3)	44 (9.0)	
5 to 14 years	86 (3.8)	56 (3.4)	30 (4.9)	0.057	77 (4.1)	29 (5.9)	0.023
Season of recruitment							
Sep-Dec	739 (32.7)	554 (33.4)	185 (30.6)		648 (34.6)	136 (27.8)	
Jan to Apr (rain)	783 (34.6)	521 (31.4)	262 (43.3)		587 (31.3)	222 (45.4)	
May-Aug	738 (32.7)	580 (35.0)	158 (26.1)	<0.001	639 (34.1)	131 (26.8)	<0.001
HIV Positive[4]	120 (5.6)	65 (4.2)	55 (9.8)	<0.001	80 (4.6)	48 (10.6)	<0.001
Weight for age <2 SD[4]	449 (20.9)	325 (20.2)	124 (22.9)	0.169	353 (20.5)	98 (22.4)	0.370
Malaria positive[4]	78 (3.5)	47 (2.9)	31 (5.3)	0.007	52 (2.9)	27 (5.6)	0.006
RSV PCR positive[4]	220 (11.9)	130 (9.4)	90 (19.9)	<0.001	146 (9.9)	74 (20.9)	<0.001
Influenza PCR positive	258 (11.4)	199 (12.0)	59 (9.8)	0.133	217 (11.6)	50 (10.2)	0.399
Year[3,5]							
2011	25 (8.8)	10 (7.3)	15 (9.3)	0.531	11 (6.1)	14 (11.8)	0.079
2012	30 (6.2)	28 (6.7)	3 (2.8)	0.121	29 (6.5)	2 (2.5)	0.167
2013	141 (16.2)	111 (15.6)	30 (19.5)	0.229	117 (15.8)	24 (18.6)	0.431
2014	70 (10.5)	59 (12.0)	11 (6.0)	0.024	60 (11.8)	10 (6.1)	0.040
Type/Subtype							

Influenza A							
H1N1pdm09	44 (2.0)	25 (1.5)	19 (3.1)		28 (1.5)	18 (3.7)	
H3N2	106 (4.7)	90 (5.4)	16 (2.6)		101 (5.4)	11 (2.3)	
A (Unsubtyped)	4 (0.2)	3 (0.2)	1 (<0.1)		3 (0.2)	1 (0.2)	
Influenza B	101 (4.3)	81 (4.9)	20 (3.3)		85 (4.5)	17 (3.5)	
Influenza A & B	3 (0.1)	0 (0)	1 (<0.1)		0 (0)	3 (0.6)	
Clinical features[4]							
Recorded fever	1048 (46.4)	618 (37.3)	430 (71.1)	<0.001	708 (39.9)	340 (69.5)	<0.001
Fast breathing	1805 (79.8)	1318 (79.6)	487 (80.5)	0.652	1398 (78.9)	407 (83.2)	0.036
Nasal flaring	569 (25.2)	167 (10.1)	402 (66.5)	<0.001	230 (12.9)	339 (69.3)	<0.001
Vomiting/ diarrhoea	392 (17.4)	264 (15.9)	128 (21.2)	0.004	287 (16.2)	105 (21.5)	0.007

1. SARI with warning signs determined in 2260 patients with documented clinical severity and hospitalisation status
2. P-values of difference between SARI with warning signs and SARI without warning signs, and between hospitalised and non-hospitalised SARI
3. Percentages represent factor column totals, or the per cent of all SARI cases assessed for the factor; for influenza by year percentages represent per cent of column total within year
4. HIV was measured in 2143 patients; weight-for-age Z score was measured in 2122 patients aged 3 to 59 months; malaria was measured in 2239 patients; RSV was measured in 1835 patients recruited from 2011-2013;
5. Fisher's exact test used to compare yearly influenza prevalence by clinical severity and hospitalisation status

Table 2. Matrix of mono and co-detection of viral pathogens by multiplex PCR in 1835 paediatric severe acute respiratory illness (SARI) cases in Blantyre, Malawi, 2011-2014[1]

	Influenza A (H3N2)	Influenza B	Influenza A (H1N1) pdm09	Influenza C	Boca-virus	Corona-virus 229	Corona-virus 43	Coronavirus 63	Entero-viruses	Adeno-virus	Human meta-pnuemo virus	Para-influenza virus 1	Para-influenza virus 2	Para-influenza virus 3	Para-influenza virus 4	Parecho-viruses	RSV	Rhino-virus
Influenza A (H3N2)	66																	
Influenza B	0	38																
Influenza A (H1N1)pdm09	1	1	32															
Adenovirus	0	0	0	9														
Bocavirus	4	4	0	0	49													
Coronavirus 229	0	1	0	0	1	5												
Coronavirus 43	7	0	1	0	15	3	38											
Coronavirus 63	2	2	0	0	3	2	5	16										
Enteroviruses	1	3	1	1	5	1	5	3	13									
Influenza C	8	3	1	4	15	2	6	3	15	77								
Human metapnuemo virus	1	3	0	0	13	0	5	1	3	13	64							
Parainfluenza virus 1	0	0	0	1	3	0	0	0	0	2	3	39						
Parainfluenza virus 2	0	1	0	0	3	0	1	1	0	2	4	2	14					
Parainfluenza virus 3	3	0	0	0	8	1	2	8	6	6	1	3	5	91				
Parainfluenza virus 4	0	1	0	0	2	0	2	0	3	4	3	1	3	5	24			
Parechovirus	3	1	1	19	12	7	0	3	2	41	0	3	2	9	3	6		
RSV	2	6	9	1	11	1	7	4	5	11	7	5	1	2	2	13	155	
Rhinoviruses	4	7	1	7	31	5	9	10	37	28	16	6	5	20	8	11	10	212
Positive tests[1]	93	64	44	19	130	17	85	48	64	162	112	56	29	142	42	86	220	358
N (%)	(5.1)	(3.5)	(2.4)	(1.0)	(7.1)	(0.9)	(4.6)	(2.6)	(3.5)	(8.8)	(6.1)	(3.1)	(1.6)	(7.7)	(2.3)	(4.7)	(12.0)	(19.5)
%Co-viral detection[2]	29.0%	40.6%	27.3%	52.5%	62.3%	70.6%	55.3%	66.7%	79.7%	52.6%	42.9%	30.4%	51.7%	35.9%	42.9%	93.2%	29.5%	40.8%

1. Represents number of positive tests among all SARI cases tested. Columns do not add up to total positive tests due to detection of multiple virus in some samples; diagonal of matrix represents mono-infection
2. Represents proportion of co-viral detection among SARI cases testing positive for the pathogen (listed at column heading)

Table 3. Demographic, seasonal and pathogen factors associated with influenza-positive severe acute respiratory illness (SARI) in children, Blantyre Malawi, 2011-2014

				Univariate			Multivariate		
	All	Influenza Negative N (%)[1]	Influenza Positive N (%)	RR[2]	95% CI	p-value	aRR[3]	95% CI	p-value
Total	2239	1990	249						
Gender									
Male	1187 (53.0)	1069 (53.7)	118 (47.4)	Ref			Ref		
Female	1052 (46.9)	921 (46.3)	131 (52.6)	1.4	1.1, 1.9	0.022	1.3	0.9, 1.8	0.069
Age									
3 to <6 months	269 (12.0)	250 (12.6)	19 (7.6)	Ref			Ref		
6 to <12 months	576 (25.7)	536 (26.9)	40 (16.1)	0.9	0.5, 1.6	0.615	0.9	0.4, 1.8	0.959
12 to <36 months	1071 (47.8)	943 (47.4)	128 (51.4)	1.6	0.9, 2.8	0.084	1.7	1.1, 2.9	0.046
36 to <60 months	241 (10.8)	198 (9.9)	43 (17.3)	3.0	1.6, 5.6	<0.001	2.9	1.6, 5.5	<0.001
5 to <15 years	82 (3.7)	63 (3.2)	19 (7.6)	2.9	1.3, 6.3	<0.001	2.9	1.3, 6.5	<0.001
Year of recruitment									
2011	272 (12.1)	248 (12.5)	24 (9.6)	Ref					
2012 (vs. 2011)	489 (21.8)	459 (23.1)	30 (12.0)	0.5	0.1, 1.6	0.228			
2013 (vs. 2011)	811 (36.2)	686 (34.7)	125 (50.2)	2.4	0.8, 7.5	0.139			
2014 (vs. 2011)	667 (29.8)	597 (30.0)	70 (28.1)	3.2	1.3, 13.3	0.015			
Season of recruitment									
Sep to Dec	726 (32.4)	648 (32.6)	78 (31.3)	Ref			Ref		
Jan to Apr (rain)	773 (34.5)	654 (32.8)	119 (47.8)	2.7	1.6, 4.4	<0.001	3.3	1.9, 5.4	<0.001
May to Aug (cool dry)[4]	740 (33.1)	688 (34.6)	52 (20.9)	1.6	0.9, 2.8	0.077	2.1	1.2, 3.6	0.009
HIV Positive[5]									
Negative	1973 (94.3)	1747 (94.2)	226 (95.4)	Ref					
Positive	119 (5.7)	108 (5.8)	11 (4.6)	0.9	0.4 1.7	0.677			
Weight for age Z score <2[5]									
No	1990 (93.2)	1766 (93.2)	224 (92.9)	Ref					

Yes	145 (6.8)	128 (6.8)	17 (7.1)	1.2	0.8,	1.6	0.364				
Malaria											
Negative	2160 (96.5)	1913 (96.1)	247 (99.2)	Ref				Ref			
Positive	79 (3.5)	77 (3.9)	2 (0.8)	0.2	0.1,	0.9	0.030	0.2	0.0	0.8	0.028
Hospitalised											
No	1750 (78.8)	1549 (77.8)	201 (80.7)	Ref							
Yes	489 (22.0)	441 (22.2)	48(19.3)	0.8	0.5,	1.1	0.180				
Blood oxygen saturation <90											
No	2291 (93.1)	2029 (96.8)	262 (98.1)	Ref							
Yes	72 (6.9)	67 (3.2)	5 (1.9)	0.7	0.3,	1.8	0.420				

1. Percentages represent column per cent of column total within each factor
2. Univariate relative risks from models that included only variable of interest and patient-level kernel smoothing factors to remove auto-correlation in residuals
3. Adjusted relative risks from a multivariable model developed using backwards selection of factors significant at p-value<0.05, and a priori inclusion of age and gender. Model included age, gender, season of recruitment, malaria status and patient-level kernel smoothing factors to remove auto-correlation in residuals
4. Risk of influenza positive SARI was significantly higher in the rainy season (January to April) compared to the cool dry season (May to August) (aRR:; 1.59, 95% CI: 1.04, 2.45)
5. HIV was measured in 2097 patients, weight-for-age Z score was measured in 2135 patients aged 3 to 59 months

Appendix –CONSORT diagram

Table 4. Incidence of severe acute respiratory illness (SARI) in children residing in Blantyre City, Malawi, by SARI type, age and HIV status[1]

Age group	HIV Un-infected: Incidence per 10,000	95% CI		HIV infected: Incidence per 10,000	95% CI		IRR_{HIV}	95% CI	
All Recruited SARI									
<1 year	89.5	85.8	93.0	155.3	127.3	191.1	1.7	1.41	2.14
12-59 months	35.8	34.9	36.9	73.3	64.7	87.8	2.0	1.82	2.44
5-9 years	1.3	1.0	1.6	16.0	9.9	24.2	12.6	7.69	19.21
10-14 years	0.8	0.7	1.0	7.9	5.5	12.7	9.6	6.52	17.10
SARI with warning signs									
<1 year	16.5	15.2	18.0	43.1	29.4	60.7	2.6	1.66	3.61
12-59 months	7.2	6.7	7.5	30.1	24.4	40.3	4.2	3.34	5.91
5-9 years	0.4	0.3	0.5	9.0	6.0	15.6	24.3	13.51	51.03
10-14 years	0.1	0.1	0.2	3.3	1.4	6.7	37.7	11.10	93.21
Hospitalized SARI									
<1 year	12.3	11.1	13.8	25.9	14.9	37.2	2.1	1.1	3.0
12-59 months	5.4	4.9	5.7	21.9	16.7	30.1	4.1	3.0	5.9
5-9 years	0.3	0.2	0.4	6.0	2.9	11.0	21.3	9.2	48.7
10-14 years	0.1	0.0	0.2	3.3	0.7	5.6	37.7	11.1	109.9
Influenza Positive SARI									
<1 year	6.3	5.3	7.6	6.5	2.2	15.4	1.0	0.40	2.51
12-59 months	4.9	4.6	5.2	3.7	1.5	8.5	0.7	0.30	1.79
5-9 years	0.3	0.2	0.4	6.0	2.0	11.8	21.3	6.76	42.07
10-14 years	0.2	0.2	0.4	0.9	0.2	2.1	8.1	2.79	19.74
RSV Positive SARI									
<1 year	17.3	16.2	19.3	17.3	8.4	29.2	0.9	0.5	1.7
12-59 months	3.2	2.9	3.3	4.9	2.0	9.3	1.5	0.6	3.0
5-9 years	0.1	0.1	0.2	0.0	0.0	0.0	0.0	0.0	0.0
10-14 years	0.0	0.0	0.0	1.8	0.9	4.6	--[2]	--	--

1. Analyses based on 131 HIV infected SARI cases, 53 HIV infected cases of SARI with warning signs, 48 HIV infected hospitalized SARI cases, 11 HIV infected influenza positive SARI cases and 13 HIV infected RSV positive SARI cases
2. Inestimable

Appendix –CONSORT diagram

Table 5. Demographic, seasonal and pathogen factors associated with SARI with warning signs in children, Blantyre Malawi, 2011-2013

				Univariate				Multivariate			
	Total N(%)[1]	SARI with warning signs N(%)	SARI without warning signs N(%)	RR[2]	95% CI		p-value	aRR[3]	95% CI		p-value
	1505	1115	390								
Gender											
Male	820 (54.5)	603 (54.1)	217 (55.6)								
Female	685 (45.5)	512 (45.9)	173 (44.4)	0.83	0.65	1.07	0.157	0.80	0.62	1.04	0.091
Age											
3 to <6 months	171 (11.3)	137 (12.3)	34 (8.7)	Ref				Ref			
6 to <12 months	390 (25.9)	294 (26.4)	96 (24.6)	1.1	0.7	1.9	0.575	1.1	0.7	1.8	0.723
12 to <36 months	720 (47.8)	525 (47.1)	195 (50.0)	1.2	0.8	2.0	0.261	1.4	0.9	2.2	0.188
36 to <60 months	164 (10.9)	122 (10.9)	42 (10.8)	1.2	0.7	2.1	0.553	1.2	0.6	2.2	0.524
5 to <15 years	60 (3.9)	37 (3.3)	23 (5.9)	1.5	0.7	3.2	0.300	1.5	0.6	3.1	0.322
Year of recruitment											
2011	248 (16.5)	105 (9.4)	143 (36.7)								
2012 (vs. 2011)	464 (30.8)	361 (32.4)	103 (26.4)	0.9	0.4	2.2	0.801				
2013 (vs. 2011)	793 (52.7)	649 (58.2)	144 (36.9)	0.9	0.4	2.3	0.820				
Season of recruitment											
Sep to Dec	572 (38.0)	445 (39.9)	127 (32.6)	Ref							
Jan to April (rain)	482 (32.0)	386 (34.6)	96 (24.6)	2.9	1.7	4.8	<.0001	2.4	1.6	3.8	<0.001
May to August (cool dry)	451 (29.9)	284 (25.5)	167 (42.8)	0.9	0.6	1.2	0.461	0.8	0.59	1.2	0.319
HIV Positive	94 (6.2)	53 (4.8)	41 (10.5)	1.9	1.2	3.0	0.008	2.4	1.4	3.9	<0.001
Mid-Upper Arm Circumference <11.5	17 (1.1)	13 (1.2)	5 (1.3)	1.2	0.5	2.8	0.706				

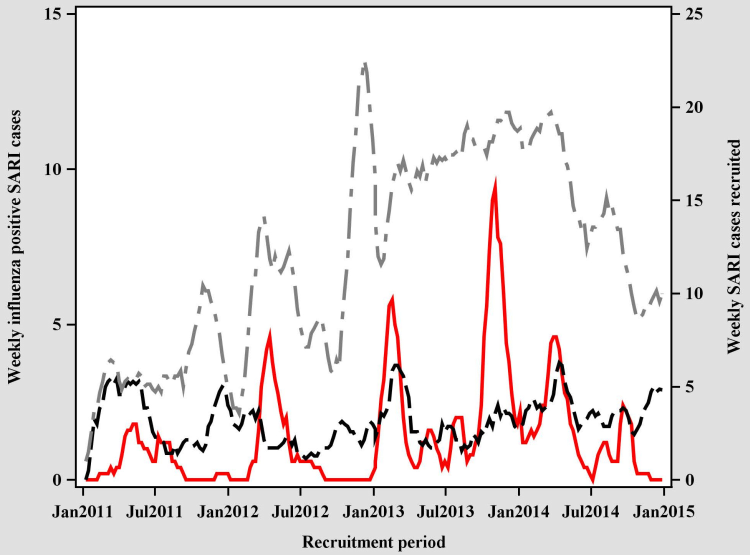
Appendix –CONSORT diagram

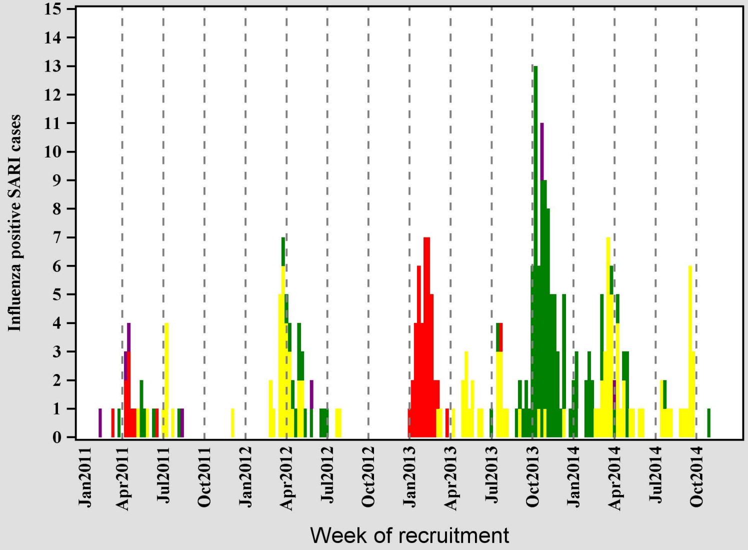
Weight for age Z score<3	73 (4.9)	52 (4.7)	21 (5.4)	1.3	0.8	2.2	0.314				
Influenza											
Negative	1332 (88.5)	986 (88.4)	346 (88.7)	Ref							
A- not subtyped/mixed	6 (0.0)	4 (0.0)	2 (0.0)	2.1	0.4	12.0	0.413				
A(H3N2)	74 (4.9)	62 (5.6)	12 (3.1)	0.6	0.3	1.3	0.207				
A(H1N1)pdm09	41 (2.7)	24 (2.2)	17 (4.4)	1.9	0.9	4.2	0.642				
B	52 (3.5)	41 (3.7)	11 (2.8)	0.9	0.5	2.1	0.978				
Co-viral detection[4]	309	214 (19.2)	95 (24.4)	1.1	0.8	1.3	0.375				
PCR Positive											
Influenza C	17 (1.1)	14 (1.3)	3 (0.7)	0.6	0.2	2.2	0.469				
Parainfluenza 1	52 (3.5)	41 (3.7)	11 (2.8)	0.8	0.4	1.5	0.427				
Parainfluenza 2	29 (1.9)	20 (1.8)	9 (2.3)	1.3	0.6	2.9	0.526				
Parainfluenza 3	127 (8.4)	95 (8.5)	32 (8.2)	0.9	0.6	1.5	0.849				
Parainfluenza 4	38 (2.5)	29 (2.6)	9 (2.3)	0.9	0.4	1.9	0.751				
RSV (A&B)	164 (10.9)	94 (8.4)	70 (17.9)	2.6	1.9	3.6	<.0001	1.9	1.3	3.0	0.002
Adenovirus	136 (9.0)	97 (8.7)	39 (10.0)	1.2	0.8	1.7	0.659				
Enterovirus	50 (3.3)	38 (3.4)	12 (3.1)	0.9	0.5	1.7	0.754				
Rhinovirus	301 (20.0)	224 (20.1)	77 (19.7)	0.9	0.7	1.4	0.895				
Bocavirus	102 (6.8)	71 (6.4)	31 (7.9)	1.2	0.8	1.9	0.286				
Coronavirus 43	66 (4.4)	43 (3.9)	23 (5.9)	1.5	0.9	2.6	0.092				
Coronavirus 63	48 (3.2)	40 (3.6)	8 (2.1)	0.6	0.3	1.2	0.142	0.2	0.07	0.70	0.010
Coronavirus 229	16 (1.1)	11 (0.9)	5 (1.3)	1.3	0.5	3.7	0.625				

Appendix –CONSORT diagram

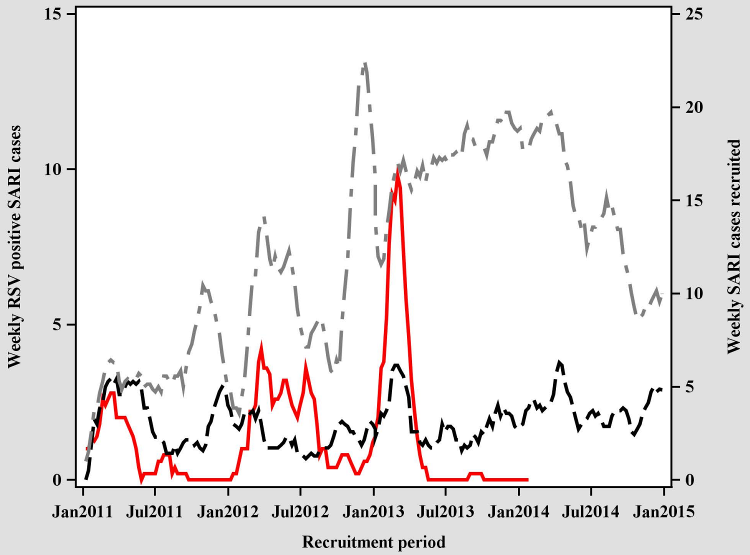
Human metapneumovirus	25 (1.7)	19 (1.7)	6 (1.5)	0.9	0.5	1.4	0.529				
Parechovirus	74 (4.9)	56 (5.0)	18 (4.6)	0.9	0.5	1.6	0.634				
Malaria Positive	42 (2.8)	24 (2.2)	18 (4.6)	2.2	1.1	4.6	0.025	2.2	1.1	4.6	0.029

1. Percentages represent factor column totals, or per cent of all SARI cases assessed for the factor
2. Univariate relative risks from models that included only variable of interest and patient-level kernel smoothing factors to remove auto-correlation in residuals
3. Adjusted relative risks from a multivariable model developed using backwards selection of factors significant at p-value<0.05, and a priori inclusion of age, and gender. Model included age, gender, season of recruitment, HIV, RSV, Coronavirus 43, malaria status and patient-level kernel smoothing factors to remove auto-correlation in residuals
4. 362/1835 (19.7%) of all SARI cases with multiplex PCR data had viral co-detection


15
10
5
0
25
20
15
10
5
0
Weekly influenza positive SARI cases
Weekly SARI cases recruited
Jan2011
Jul2011
Jan2012
Jul2012
Jan2013
Jul2013
Jan2014
Jul2014
Jan2015
Recruitment period



Influenza positive SARI cases
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
Jan2011
Apr2011
Jul2011
Oct2011
Jan2012
Apr2012
Jul2012
Oct2012
Jan2013
Apr2013
Jul2013
Oct2013
Jan2014
Apr2014
Jul2014
Oct2014
Week of recruitment



15
10
5
0
25
20
15
10
5
0
Weekly RSV positive SARI cases
Weekly SARI cases recruited
Jan2011
Jul2011
Jan2012
Jul2012
Jan2013
Jul2013
Jan2014
Jul2014
Jan2015
Recruitment period