RSV infection in children hospitalised with severe lower respiratory tract infection at the Red Cross War Memorial Children's Hospital (2012-2013)

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(Minor dissertation)

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Acknowledgements

This dissertation takes the form of a publication ready format. The manuscript will be submitted for publication. This research has been written according to the format required by the Plos One journal. The format required for submission can be found in Appendix 3.

I would like to thank my supervisor, Prof Rudzani Muloiwa and co-supervisor, Prof Heloise Buys for their constant support, guidance, encouragement and patience throughout this journey. This work would not have been possible without them. It has been a privilege to work closely with them both and I have learnt so much from their wealth of knowledge both in paediatrics and in research.

This MMED used an already existing database from a parent study done in 2016. I would like to acknowledge Prof Rudzani Muloiwa who piloted this parent study and undertook the data collection. The parent study has been referenced in the text of this research under the 'Materials and methods' section.

Abbreviations

(in order as they appear in the text)

RSV	Respiratory syncytial virus
LRTI	Lower respiratory tract infection
NP	Nasopharyngeal swab
IS	Induced sputum
LMIC	Low- and middle-income country
HIV	Human immunodeficiency virus
PCR	Polymerase chain reaction
RCWMCH	Red Cross War Memorial Children's Hospital
WHO	World Health Organization
ART	Antiretroviral therapy
ELISA	Enzyme-linked immunosorbent assay
HEU	HIV exposed uninfected
HUU	HIV unexposed uninfected
NWFA	Normal weight for age
UWFA	Under weight for age
ICU	Intensive care unit
COVID-19	SARS-CoV2 disease

Abstract

Objectives: Low- and middle-income countries carry the largest burden of Respiratory syncytial virus (RSV) disease, with most deaths occurring in these settings. This study aimed to investigate the burden of RSV disease in South African children hospitalised with lower respiratory tract infection (LRTI), with specific reference to incidence, risk factors, and co-infections.

Methods: A database from a previous prospective study containing demographic, laboratory and clinical data on children hospitalised with LRTIs in Cape Town, South Africa, was used. A nasopharyngeal (NP) swab and induced sputum (IS) were tested for RSV PCR. Descriptive statistics were used to characterise the study population, and a multivariable analysis of risk factors and co-infections was done.

Results: RSV was detected in 142 (30.9%; 95%CI 26.7-35.3) of the included 460 study children with LRTI. The median age of RSV-positive children was 4.6 (IQR 2.4-9.7) months compared to RSV-negative children of 10.5 (IQR 4.4-21.3) months, P = <0.001. Most cases occurred in autumn and winter with 126 (89%) cases over this period. IS demonstrated greater sensitivity for RSV diagnosis with 135 cases (95.1%) detected on IS and 57 cases (40.1%) identified on NP; P<0.001. The median length of hospital stay was 3.3 (SD 4.2) days in the RSV positive group and 2.7 (SD 3.3) days in the RSV negative group; P<0.001.

The number of detected viral pathogens was a median of 1 (IQR 0-2) in RSV positive children (when RSV was excluded from the count) compared to 2 (IQR 2-3) in RSV negative children; P<0.001. The presence of RSV was independently associated with a reduction in the frequency of most viruses tested for on PCR.

Conclusions: RSV is common in children hospitalised with LRTI and mainly affects younger children. There is an urgent need to find an effective vaccine to prevent RSV pneumonia in children worldwide, especially in LMICs that carry the greatest burden of disease.

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Key words: RSV, children, African, nasopharyngeal swab, induced sputum, molecular diagnostic testing

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Conclusions: RSV is common in children hospitalised with LRTI and mainly affects younger children. There is an urgent need to find an effective vaccine to prevent RSV pneumonia in children worldwide, especially in LMICs that carry the greatest burden of disease.

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Introduction

Lower respiratory tract infections (LRTIs) are a leading cause of death in children worldwide. Following the implementation of successful vaccination programs, the causative pathogens have changed with viruses emerging to dominate over bacterial causes [1]. The world has seen a substantial reduction in LRTI deaths with mortalities having halved over the last twenty years. However, there is still much progress to be made, specifically in low- and middle-income countries (LMICs) [2]. The incidence of Respiratory syncytial virus (RSV) associated pneumonia is similar globally, but LMICs carry 99% of the burden of RSV disease-related mortality [3].

In addition to mortality, RSV causes significant morbidity in childhood. Almost all of the world's children will be infected with RSV by their second birthday. Repeated infections may occur, as RSV infection does not seem to confer protective immunity against subsequent infections [4, 5] . A study done in the Western Cape province of South Africa demonstrated that LRTIs in early infancy may be associated with decreased lung function at the age of 1 year [6]. This impaired lung function may persist through childhood into adulthood and if it did, could lead to an increased risk of recurrent respiratory infections and chronic respiratory disease later on in life [7, 8].

Universally, the majority of severe RSV disease occurs in younger children, mostly under the age of one. Although most children admitted to hospital with RSV pneumonia are previously healthy, risk factors for severe disease have been identified [1]. There is an increased risk of severe disease in babies born prematurely and in those with pre-existing cardiopulmonary disease [5]. Malnutrition and HIV, especially relevant in LMICs such as South Africa, have been found to predispose children to severe disease [5, 9]. The 2016 Global Burden of

Disease Study, conducted in the same year as our study, found that childhood wasting was present in 60% of deaths associated with LRTIs in the under 5 age group [10]

RSV is estimated to cause 33 million LRTIs per annum and 3.4 million hospitalisations in children under the age of 5 years worldwide [11]. RSV infection occurs in distinct seasonal patterns worldwide. In South Africa, RSV infection shows a predilection for the autumn and winter months [12]. Recent reports have highlighted the role of RSV outbreaks in disrupting health services in both high income and LMIC settings. This has placed a huge burden on health systems and has had significant cost implications for countries around the world [13, 14].

In South Africa, as in most LMICs, RSV infections are rarely confirmed by laboratory diagnosis with diagnoses most often being made clinically, making the burden of RSV disease difficult to assess [15]. However, understanding trends of the burden of RSV disease will assist in prioritising interventions and help track progress towards global goals in both the prevention and treatment of LRTIs [9].

There is currently no specific treatment for RSV disease and management is largely supportive [5]. This places a huge burden on health systems and has significant cost implications for countries around the world. The prevention of severe disease has therefore become a global target strategy. There is currently no registered RSV vaccine available worldwide. Monoclonal antibodies, such as palivizumab, have shown promise in vulnerable children but the dosing logistics are impractical and the costs unaffordable to most LMICs [16].

This study aimed to investigate the burden of RSV disease at a tertiary children's hospital in Cape Town, South Africa. We aimed to determine the prevalence of PCR confirmed infection, risk factors for severe disease, and associated co-infections over a one year period. Secondarily, the study sought to assess appropriate method of diagnosis. As other studies suggested that IS and NP may have different abilities to identify pathogens, the study sought to secondarily assess the best sample to be used for diagnostic confirmation [17].

Materials and methods

Study population

The current study is a sub-study of the one previously published in 2016 [17]. It uses existing data collected prospectively over a one-year period between September 2012 and September 2013 at the Red Cross War Memorial Children's Hospital (RCWMCH) in Cape Town, South Africa. Briefly, the study includes 460 children, aged less than 13 years who were admitted to the short stay paediatric ward with an acute respiratory illness. Inclusion criteria were WHO-defined age-specific tachypnoea or lower chest wall indrawing, apnoea, age less than 13 years, informed parental consent and enrolment into the study within 48 hours of admission. A maximum of 4 children were conveniently enrolled per working day to ensure even distribution of enrolment throughout the year. Participants were excluded if they had a previous admission to a healthcare facility in the preceding two weeks. We used the WHO 'severe acute respiratory infection' definition when referring to severe disease - 'an acute respiratory illness with a history of fever or measured fever of ≥ 38 °C and cough, with onset within the past 10 days requiring hospitalisation' [18]. All children were managed according to national and departmental guidelines and were followed up until discharge from hospital. **Figure 1**

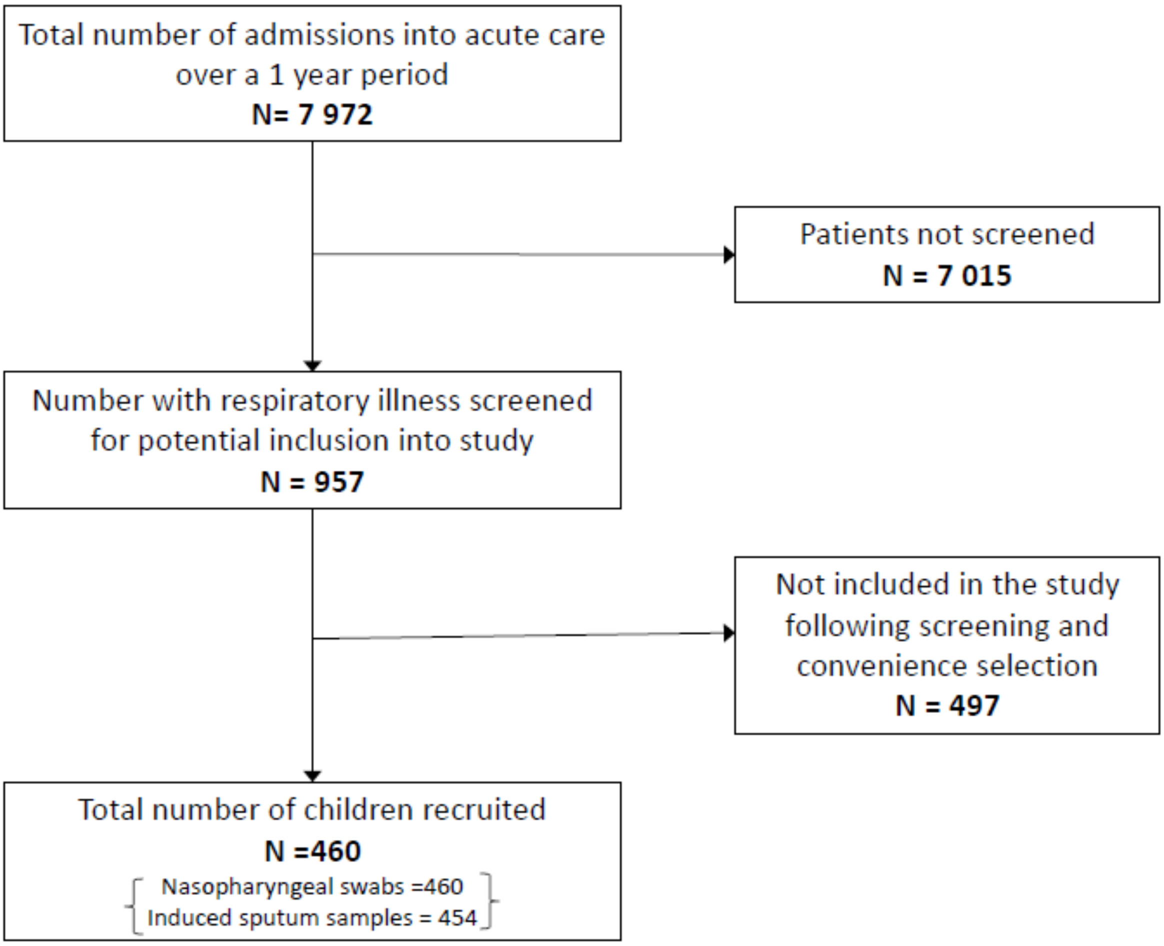

Figure 1: Flow diagram of participants in the study

Procedures

The study was conducted at RCWMCH, a public referral hospital catering for children outside the neonatal period aged up to 13 years. Caregivers of all participants were interviewed for clinical information and patient outcomes were obtained from clinical notes. The Road to Health card, an individualised handheld record, was reviewed to provide information regarding birth history, immunization status and growth. The nutritional status of each child was classified according to WHO weight-for-age Z-score criteria as normal (above -1 Z score), mildly (between -1 and -2 Z score)-, moderately (between -2 and -3 Z score)- or severely (less than -3 Z score)- underweight-for-age [19]. History of HIV exposure, infection and antiretroviral therapy (ART) were also noted. All children were tested for HIV. HIV infection was confirmed with an HIV PCR test in children less than 18 months old, or with two ELISA tests in children older than 18 months. Children were classified as 'HIV infected', 'HIV-exposed uninfected (HEU)' or 'HIV- unexposed uninfected (HUU)'. A physical examination was performed on each child and oxygen saturations were measured and recorded.

In order to obtain microbiologic confirmation of RSV infection, a nasopharyngeal swab (NP) and an induced sputum (IS) were sequentially performed. IS was collected after nebulisation with a bronchodilator and hypertonic saline. Specimens were then sent to the laboratory for PCR testing on a multiplex real-time PCR assay, the FTD® Resp 33 (Fast-Track Diagnostics, Esch-sur-Alzette, Luxembourg). The assay is able to identify presence of a range of viruses and bacteria.

Ethical approval

Ethical approval for this study was granted by the Human Research Ethics Committee of the University of Cape Town Faculty of Health Sciences, HREC 726/2019.

Statistical analysis

Baseline descriptive statistics were used to characterise the study population. Percentages (%) and their 95% confidence intervals (95% CI) were used to depict proportions of categorical variables, while medians and interquartile ranges (IQR), or means with standard deviations (SD) summarised all continuous variables, as appropriate. The strength of association between two categorical variables was tested for using the $\chi 2$ or Fisher's exact test. The Mann-Whitney U test was used to assess strength of association between continuous variables.

Potential risk factors for PCR confirmed RSV on IS were assessed. The variables assessed included age group of the patient, HIV and nutritional status, crèche attendance, household cigarette smoking, and history of breastfeeding. Relative risk was used to quantify the measure of effect between the RSV negative and RSV positive groups with respect to risk factors. Further analysis assessed the association between presence of RSV and presence of other respiratory pathogens in the IS sample using univariate and multivariable modelling. In all instances, generalised linear modelling using Poisson regression with robust error variance estimated adjusted relative risks (aRR) and their 95% confidence intervals. A significance level of P<0.05 was used for all analyses.

While the study analysed all 460 participants in describing the cohort and overall prevalence of confirmed RSV, assessment of risk and co-infections was limited to induced sputum samples. For the assessment of risk and co-infection IS sample with the 135 RSV cases detected on IS were used in the analysis. We assumed for the purpose of this study that RSV found in the IS (which accounted for 95% of cases) was most indicative of lower respiratory tract involvement. Other studies have also noted the challenges of determining pneumonia aetiology using specimens obtained from the upper respiratory tract. Whereas induced sputum allows for the expulsion of expectorated sputum from below the vocal cords (i.e., the 'lower respiratory tract'), other procedures such as NP swabs are thought to collect organisms present in the upper respiratory tract. A positive finding from the upper respiratory tract may indicate a live pathogen, an infection or colonisation of the upper tract only or residual DNA from a previous infection. It is therefore difficult to prove causality of lung disease from the detection of an organism in the upper respiratory tract. Hence, we assumed for the purpose of this study that RSV found in the IS was most indicative of lower respiratory tract involvement [20-22].

Results

Baseline characteristics

The median age of the 460 children included was 7.8 (IQR 3.6-18.0) months and 258 (56.1%) were male. HIV status was determined to be HUU in 349 (75.9%), HEU in 92 (20.0%), whilst 19 (4.1%) were HIV infected. Of the HIV infected children, nine (47.4%) were on antiretroviral therapy (ART) with four having attained viral suppression. Immunisation status was known in all but nine of the enrolled children. Of the 451 with documented immunisation records, 325 (72.1%) had received immunisations appropriate for age at the time of admission. Nutrition status was recorded in all children with 415 (90.2%) children having normal weight-for-age (NWFA) and 45 (9.8%) children being classified as mildly to severely underweight-for-age (UWFA). **Table 1**

Table 1: Baseline characteristics of the study children

BASELINE CHARACTERISTIC	Total number (N=460) n (%)	RSV positive (n=142) n (%)	RSV negative (n=318) n (%)
AGE			
< 3 months	86 (18.7)	43 (30.2)	43 (13.5)
3-11 months	203 (44.1)	68 (47.9)	135 (42.4)
12-23 months	94 (20.4)	22 (15.5)	72 (22.6)
> 23 months	77 (16.7)	9 (6.3)	68 (21.3)
SEX			
Male	258 (56)	84 (59.2)	174 (54.7)
Female	202 (43.9)	58 (40.8)	144 (45.2)
HIV STATUS			
Unexposed uninfected	349 (75.9)	108 (76.1)	241 (75.8)
Exposed uninfected	92 (20.0)	32 (22.5)	60 (18.9)
Infected	19 (4.1)	2 (1.4)	17 (5.3)
NUTRITIONAL STATUS			

Normal	351 (76.3)	116 (81.6)	235 (73.4)
Mild under-nutrition	64 (13.9)	19 (13.3)	45 (14.2)
Moderate under-nutrition	33 (7.2)	7 (4.9)	26 (8.2)
Severe under-nutrition	12 (2.6)	0 (0)	12 (3.8)
IMMUNISATIONS APPROPRIATE FOR AGE			
Up to date for age	325 (70.7)	104 (73.2)	221 (69.5)
Not up to date for age	126 (27.4))	37 (26.1)	89 (28.0)
Unknown	9 (2.0)	1 (0.7)	8 (2.5)
HOME CIGARETTE SMOKING			
No home smoker	298 (64.8)	88 (61.0)	210 (66.0)
Home smoker	162 (35.2)	54 (38.0)	108 (34.0)
CRECHE ATTENDANCE			
Creche attender	96 (20.9)	20 (14.1)	76 (23.9)
Non creche attender	364 (79.1)	122 (85.9)	242 (76.1)
HISTORY OF BREASTFEEDING			
Never breastfed	60 (13.0)	20 (14.1)	40 (12.6)
Breastfed first 4 months	323 (70.2)	103 (72.5)	220 (69.2)
Breastfed > 4 months	77 (16.7)	19 (13.3)	58 (18.2)

Confirmed RSV infection

PCR was positive for RSV in 142 (30.9%; 95%CI 26.7-35.3) of the 460 study participants. In 85 (59.9%) of the cases, PCR for RSV was detected on IS only, 7 cases (4.9%) were identified on NP swab only and 50 (35.2%) were identified on both NP and IS. **Figure 2**. Hence IS was able to identify 135 (95.1%) of the cases, while NP could identify 57 (40.1%) of the cases, P<0.001.

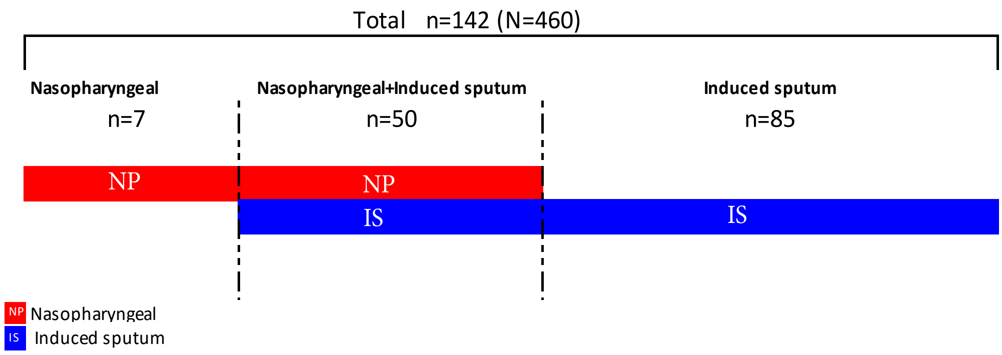

Figure 2: Number of respiratory syncytial virus (RSV) positive specimens by type of sample analysed

Most RSV cases were seen in the months of February to July, with 126 (88.7%) of the 142 cases seen over this period. The peak of the cases was seen in the months of March and April. This observed pattern mirrors the known well-established pre-COVID-19 pandemic seasonal pattern of RSV in South Africa [23]. **Figure 3**

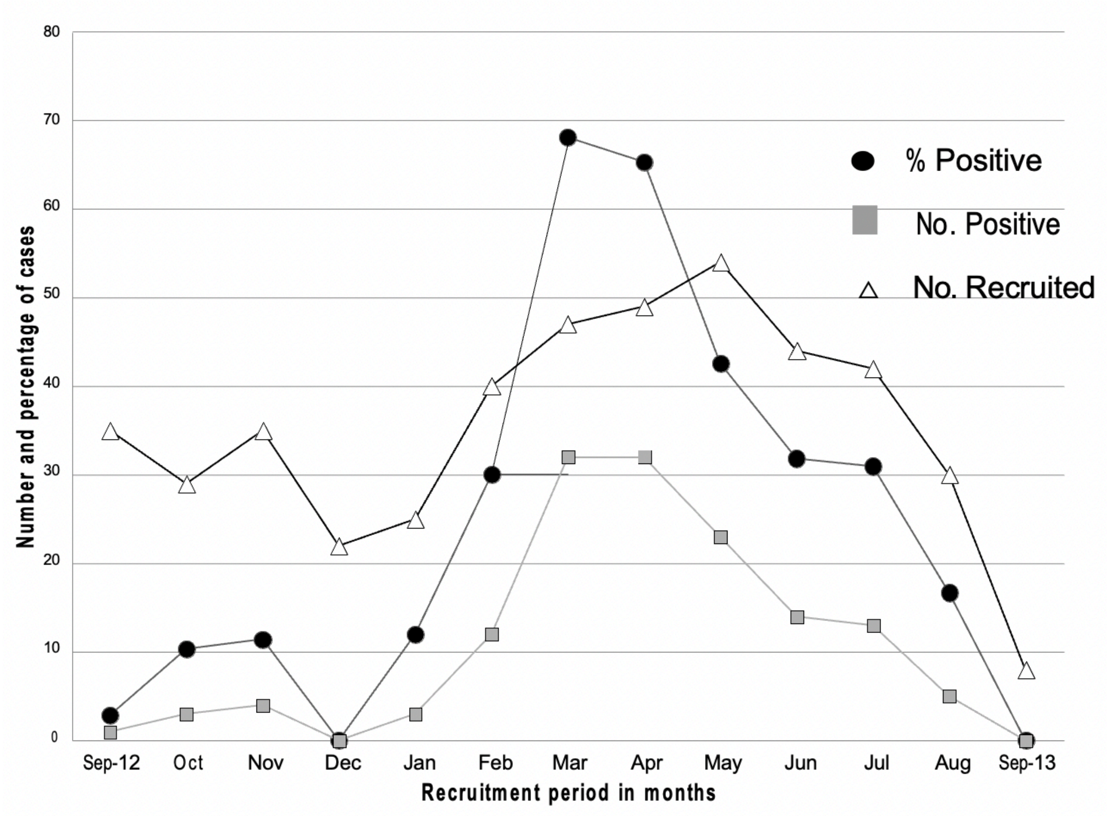

Figure 3: Percentage and number of confirmed respiratory syncytial virus cases per month

Risk factors for confirmed RSV infection

The median age of children in whom RSV was detected on IS was 4.6 (IQR 2.4-9.7) months compared to children who were RSV-negative at 10.5 (IQR 4.4-21.3) months, P<0.001. **Figure 4**. PCR for RSV was positive in 82 (32.4%) of the 253 males and in 53 (26.3%) of the 201 females, P=0.162.

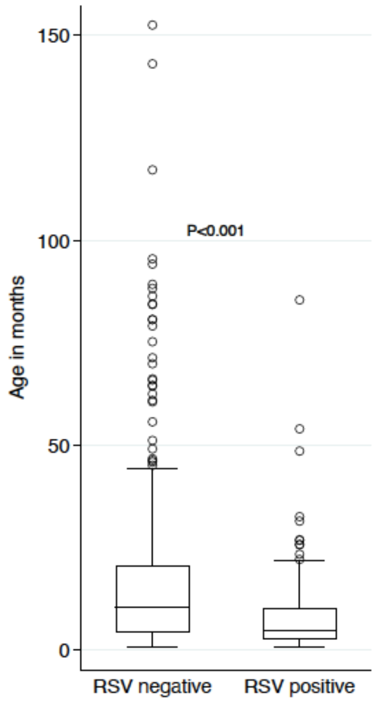

Figure 4: Ages of children by Respiratory syncytial virus (RSV) status

The proportion of children testing positive for RSV was highest in those less than 3 months of age (43/86, 50.0%) compared to those over 23 months of age (9/77, 11.7%). The frequency of RSV decreased with an increase in age category. In 110 (31.6%) of the 348 children with normal weight-for-age, PCR for RSV was positive compared to 25 (26.3%) who were classified as mild-to-moderately UWFA. The risk of RSV was similar irrespective of HIV or crèche status, exposure to household tobacco smoke, or breastfeeding history. **Table 2**

Table 2: Risk factors for confirmed respiratory syncytial virus (RSV) infection in study children (N=454)

		Relative Risk (95% Confidence Interval)	
RISK FACTOR	Risk n/N (%)	Crude	Adjusted*
AGE			
< 3 months	43/84 (51.2)	1	1
3-11 months	64/199 (32.2)	0.63 (0.47-0.84)	**0.63 (0.47-0.83)**
12-23 months	21/94 (22.3)	0.44 (0.28-0.67)	**0.44 (0.29-0.68)**
>23 months	7/77 (11.7)	0.18 (0.08-0.37)	**0.19 (0.09-0.39)**
NUTRITIONAL STATUS			
Normal	110/348 (31.6)	1	1
Mild under-nutrition	18/62 (29.0)	0.92 (0.60-1.40)	0.93 (0.60-1.43)
Moderate under-nutrition	7/33 (21.2)	0.67 (0.34-1.32)	0.67 (0.35-1.32)
Severe under-nutrition	0	NA	NA
HIV STATUS			
Unexposed uninfected	103/347 (29.7)	1	1
Exposed uninfected	30/88 (34.1)	1.15 (0.82-1.60)	1.12 (0.80-1.53)
Infected	2/19 (10.5)	0.36 (0.95-1.33)	0.48 (0.12-1.89)
CRECHE ATTENDANCE			
Non attender	113/357 (31.7)	1	1
Attender	20/95 (21.1)	0.67 (0.44-1.01)	1.00 (0.67-1.52)
BREASTFEEDING HISTORY			
Never breastfed	19/58 (32.8)	1	1
Breastfed first 4 months	98/320 (30.6)	0.94 (0.62-1.40)	0.86 (0.56-1.31)
Breastfed > 4 months	18/76 (23.7)	0.72 (0.42-1.25)	0.64 (0.36-1.12)
HOME CIGARETTE SMOKING			
No home smoker	83/294 (28.2)	1	1
Home smoker	52/160 (32.5)	1.15 (0.86-1.54)	1.15 (0.87-1.51)

Risk ratio 95% confidence Intervals that do not cross the null value of 1 are shown in **bold typeface**

*** Model adjusted for age, sex and HIV status**

Co-infection of RSV with other respiratory pathogens

The median number of detected viral pathogens was 2 (IQR 1-3) in RSV-positive children when RSV was included in the count compared to 2 (IQR 2-3) in RSV-negative children, P=0.104. When compared to the latter, the median number of viral pathogens in RSV-positive children when RSV was excluded was 1 (IQR 0-2), P<0.001. Bacterial co-infections were found in 131 (97.0%) of the 135 RSV-positive and 304 (95.3%) of 318 RSV-negative cases, P=0.608. The median number of bacterial pathogens in RSV-positive and RSV-negative children was 2 (IQR1-3) and 2 (IQR1-3) respectively, P=0.603. **Figure 5**

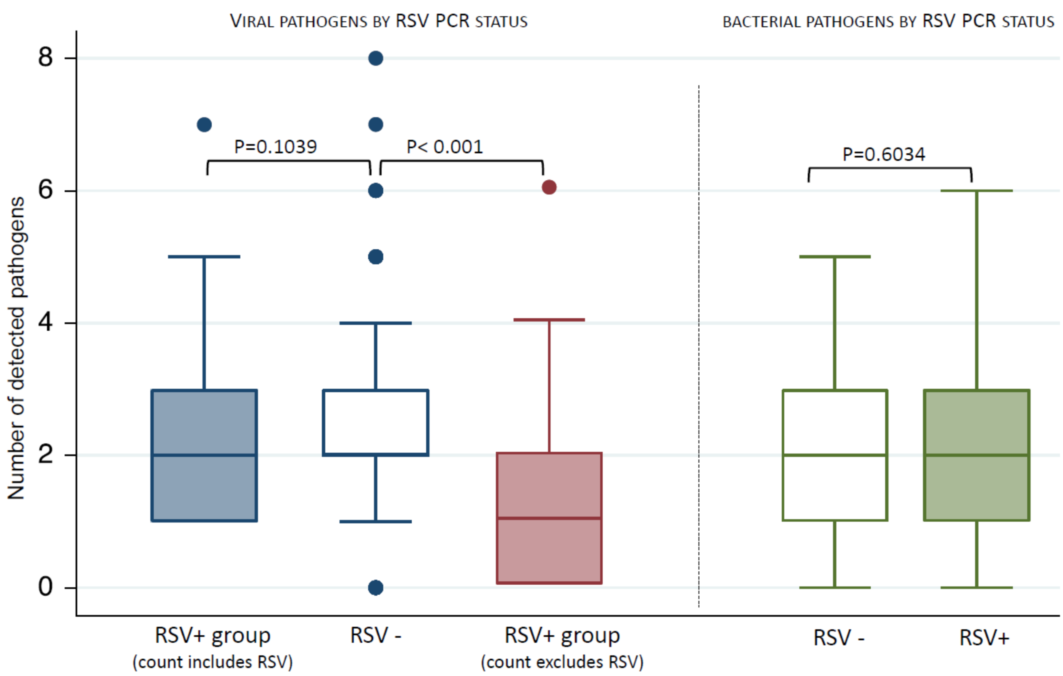

Figure 5: Number of pathogens detected on polymerase chain reaction (PCR) by Respiratory syncytial virus (RSV) status

NB: RSV positive group analysed with and without RSV included in the total count of viral pathogens

Rhinovirus was the most commonly detected virus in 34/135 (25.2%) of RSV-positive children while *Moraxella catarrhalis* was the most commonly found bacteria in 87/135 (64.4%). Overall, the frequency of other viruses was inversely associated with the presence of RSV, while the frequency of bacterial pathogens was similar in both groups of children.

Table 3

Table 3: Presence of co-pathogens by RSV status

		RSV PCR n (%)		
Pathogen[¶]	**Total n=454 n (%)**	**Positive n=135 n (%)**	**Negative n=319 n (%)**	**P value[#]**
Viral organisms				
Rhinovirus	222 (48.9)	34 (25.2)	188 (58.9)	**<0.001**
Adenovirus	117 (25.8)	9 (6.7)	108 (33.9)	**<0.001**
Enterovirus-parechovirus	80 (17.6)	9 (6.7)	71 (22.3)	**<0.001**
Bocavirus	80 (17.6)	20 (14.8)	60 (18.8)	0.347
Parainfluenza (1,2,3 4)	75 (16.5)	11 (8.1)	64 (20.1)	**<0.001**
Metapneumovirus A & B	45 (9.9)	2 (1.5)	43 (13.5)	**<0.001**
Coronavirus	35 (7.7)	11 (8.1)	24 (7.5)	0.848
Influenza (A, B, C)	28 (6.2)	4 (3.0)	24 (7.5)	**0.086**
Bacterial organisms				
Moraxella catarrhalis	295 (65.0)	87 (64.4)	208 (65.2)	0.914
Streptococcus pneumoniae	240 (52.9)	72 (53.3)	168 (52.7)	0.918
Haemophilus influenzae	231 (50.9)	61 (45.1)	170 (53.3)	0.124
Staphylococcus aureus	136 (30.0)	46 (34.0)	90 (28.2)	0.219
Haemophilus influenzae B	16 (3.5)	5 (3.7)	11 (3.4)	1.000
Mycoplasma pneumoniae	10 (2.2)	4 (3.0)	6 (1.9)	0.493
Bordetella pertussis	9 (2.0)	1 (0.74)	8 (2.5)	0.291
Chlamydia pneumoniae	7 (1.2)	2 (1.5)	5 (1.6)	1.000

IS= Induced sputum. # Two-sided Fisher's exact or Chi Square tests P-values; Bold Typeface = P < 0.1

[¶] Organisms shown in descending order of total frequency for each pathogen group

The presence of RSV was independently inversely associated with most viruses tested for on PCR after adjusting for potential confounding. **Table 4** shows relative risk of viral pathogens associated with RSV PCR status at P<0.1 after adjusting for potential confounding.

Table 4: Risk of viral pathogens associated with respiratory syncytial virus (RSV) PCR status at P<0.1

Table 4. Risk of viral pathogens associated with RSV PCR status at P<0.1

Co-infection	***RSV* PCR n (%)**		**RR (95% Confidence interval)**	
	Positive n=135	**Negative n=319**	**Crude**	**Adjusted[#]**
Rhinovirus	34(25.2)	188(58.9)	0.35(0.25-0.50)	**0.40(0.29-0.57)**
Adenovirus	9(6.7)	108(33.9)	0.21(0.11-0.39)	**0.24(0.13-0.45)**
Enterovirus-parechovirus	9(6.7)	71(22.3)	0.33(0.18-0.63)	**0.39(0.21-0.75)**
Parainfluenza	11(8.1)	64(20.1)	0.45(0.25-0.79)	**0.44(0.25-0.77)**
Metapneumovirus	2(1.5)	43(13.5)	0.14(0.03-0.53)	**0.13(0.03-0.51)**
Influenza (A,B,C)	4(3.0)	24(7.5)	0.46(0.19-1.17)	**0.48(0.19-1.17)**

RR = Relative risk; [#] Multivariable model adjusted for age in months, sex and HIV status and nutritional status; confidence intervals not overlapping the null value of 1 are shown in bold typeface.

Outcomes

None of the children with LRTI included in the study died during the study period. Supplemental oxygen was required in 22 (16.3%) of the 135 children with RSV compared to 49 (15.4%) in the non-RSV group, P= 0.958. Length of hospitalisation in the RSV group averaged 3.3 (SD 4.2) days while those in the non-RSV group stayed an average of 2.7 (SD

3.3) days, P<0.001. Admission to a high care unit or ICU was required by 3 (2.2%) of the children in the RSV group compared with 9 (2.8%) children in the non- RSV group, P=0.5.

Discussion

This study has demonstrated that RSV is a common finding in children hospitalised with LRTI in Cape Town, South Africa. A third of participants in our study had laboratory-confirmed RSV. RSV showed a seasonal pattern and was more frequently detected in younger children in the cohort. Our study indicated that IS was the sample with a higher detection rate for RSV compared to NP. Presence of RSV was significantly associated with lower frequencies of other viruses. Other than age, no risk factor for RSV infection was identified. Presence of RSV PCR was associated with an increase in the length of hospital stay for LRTI.

The prevalence of detected RSV PCR in our study increased with decreasing age with the majority of infections occurring in children aged less than one year. These findings echo that of the PERCH study, a multi-country case-control study of pneumonia aetiology in 4232 children below 5 years of age. PERCH identified RSV to be the most common pathogen overall in the aetiology of severe pneumonia with 31.1% of cases due to RSV, a similar finding to our study. Similarly, RSV infection in the PERCH study was significantly higher in infants compared to older children with the highest frequency between the ages of one and six months [2]. The Drakenstein Health Study, a multi-year South African birth cohort study, demonstrated a similarly high frequency with RSV the most identified organism occurring in 23% of LRTI cases. Again, the highest incidence of RSV occurred in children less than 6 months of age [20].

The majority of RSV-related disease in our study children occurred in otherwise healthy, well-nourished children, a finding seen worldwide. A study done including participants from other LMIC's including Nigeria, Gambia and India found no link between childhood malnutrition and RSV disease. In these studies, malnourished children demonstrated less severe disease and less RSV deaths compared to well-nourished children. This has been postulated to be due to a weaker immune response to the virus in malnourished children [24].

A multi-site study involving South African public hospitals called the Severe Acute Respiratory Virus Program (SARI), a countrywide respiratory virus surveillance program reported that HIV infection led to a 3-5-fold increased risk of hospitalisation in RSV-associated LRTI [23]. This 'viral watch' program also found that the incidence of RSV LRTI was higher in HEU children compared to those who were HUU [25] . In contrast, the present study did not demonstrate similar findings. However, the small number of children living with HIV in our study makes it impossible to do a meaningful comparison. Other factors such as crèche attendance, household smoke exposure, and breastfeeding history seem to be associated with RSV infection.

Our study found an increased median length of stay in the RSV-positive group compared to the RSV-negative group. A study done in the United Kingdom over eight successive bronchiolitis seasons in infants < 6 months old had similar findings. Detection of RSV was significantly associated with an increased average length of stay, with an average 84 hour stay in the RSV-positive group compared to a 48 hour stay in the RSV-negative group, ($P<0.001$) [26].

The previously mentioned Drakenstein Child Health study reported a higher sensitivity for IS in the diagnosis of several organisms, including RSV, compared to NP specimens [20]. The present study found the use of IS to have a significantly higher yield of detection of RSV compared to NP. RSV was more than twice likely to be identified on IS with a 95% detection rate compared to 40% in NP specimens from the same children.

In our study the presence of RSV was independently associated with a reduction in the frequency of most viruses tested for on PCR. This observation was particularly strong for adenovirus, enterovirus, rhinovirus, parainfluenza and metapneumovirus ($P<0.01$). A recent review article reported a lower-than-expected rate of co-infection between RSV and other viruses. The authors hypothesised that one virus may competitively inhibit the replication of another virus [27]. If this hypothesis is correct, it could explain our findings that RSV may potentially inhibit replication of other viruses.

The post COVID-19 period has seen a number of countries, including South Africa, struggle with RSV outbreaks that threaten the stability of the health care system [13, 28] . This phenomenon together with the burden and epidemiology of RSV disease demonstrated in this study highlights the importance of recent announcements of a potentially effective vaccine against RSV. This vaccine is intended to protect young infants through transplacental antibody transfer by immunising pregnant women [29]. This follows disappointments following failure of a previous promising candidate to reach the market [30].

Our study, from a single site, excluded children who were admitted directly to a high care or ICU setting. This may have affected our data report on disease severity, length of hospital stay and need for further respiratory support. In addition, the design of our study likely underestimated the burden of RSV disease. As only hospitalised cases were included, we missed a large number of children with respiratory disease seen in the outpatients' section of the hospital during the same period – a group likely to constitute the bulk of RSV-infected children. The exclusion of prematurity and underlying cardiopulmonary disease as risk factors in the study participants is a limitation of this study. However, with the use of RSV PCR we were able to discover the large burden of RSV disease in our setting, especially in younger children and how IS may be a more useful tool in RSV diagnosis than NP. This study done pre-COVID-19 will provide a valuable comparison for any future similar studies considered in the now 'post COVID' era with respect to any changes in the RSV epidemiology in our area. Through all these findings we can better understand the trends in the burden of RSV disease in our setting and hope this may be used to prioritise interventions in the future.

Conclusion

RSV is common in South African children hospitalised with LRTI and tends to affect younger children who require prolonged hospitalisation. Detection of RSV can potentially be improved by the use of IS specimens. Ultimately, there is an urgent need to find an effective vaccine to prevent RSV pneumonia in children, especially young infants. Once available such intervention should be made accessible to include children in LMICs that carry the greatest burden of RSV disease.

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Chapter Two: Appendices

Appendix One: Research Protocol

RESEARCH PROTOCOL

RSV infection in children hospitalised with severe lower respiratory tract infection at the Red Cross War Memorial Children's Hospital (2012-2013)

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BACKGROUND

In 2016, lower respiratory tract infections (LRTI's) caused 13,1% of all deaths in children younger than five years worldwide. Respiratory Syncytial Virus (RSV) was the second leading cause of these deaths. Globally, RSV causes over 30 million new acute lower respiratory tract infections each year, results in more than 3,4 million hospital admissions and up to 199 000 childhood deaths in the under five year old age group. Although substantial progress has been made to reduce the global burden of pneumonia, it still remains a global health problem. In the United States, around 20% of the annual birth cohort require outpatient medical attention due to RSV related illness during the first year of life. Nearly all children in the world will be infected with RSV by the age of two Repeated infections may occur as RSV infection does not protect against subsequent infections.

In terms of South African data, a national study done between 1998 and 2009 demonstrated a mean of 20 441 all respiratory deaths per year in under five-year-olds, with the mean annual RSV-associated respiratory deaths recorded as 10 per 100 000 person years. In a paediatric ICU in South Africa, 20% of children admitted with symptomatic respiratory disease had positive viral isolates, with RSV found to be present in 27,7% of cases. The majority of severe disease and RSV related deaths occur in children under the age of one. In the South African national study, it was found that mean mortality rate for all-respiratory deaths, including RSV, was 12 times higher in children under the age of one, compared to children aged one to four years. Most life-threatening RSV infections occur in previously healthy children. One study done showed up to 79% of children were previously healthy. However, there is a high case fatality rate among children with underlying conditions. In low-income or lower middle-income countries, at least 28% of deaths occurred in children with severe comorbidities.

Although the incidence of RSV associated pneumonia is similar in the world, low and middle-income countries (LMICs) carry the biggest burden of RSV disease and mortality with 99% of deaths occuring in these settings. In South Africa, as in most LMICs, RSV infections are rarely confirmed by laboratory diagnosis, making the burden of disease difficult to assess. In 2013 the World Health Organisation (WHO) in collaboration with the United Nations Children's Fund (UNICEF) started the Global Action Plan for Pneumonia and Diarrhoea (GAPPD). The initiative aims to decrease the number of and mortality from LRTI's worldwide by 2025.

Understanding trends of the burden of RSV disease is crucial to prioritise interventions and track progress towards global goals in the prevention and treatment of LRTIs.
RSV is a single stranded RNA virus responsible for acute, communicable infection of the respiratory tract Spread is mainly by close contact with infectious secretions or touching of contaminated surfaces The pathogenesis of RSV infection includes direct cellular damage from viral replication as well as an associated exaggerated host immune response. The viral infection, after an incubation period of four to six days, starts in the upper respiratory tract where viral replication in the nasal epithelium results in congestion and rhinorrhea. Infection then spreads to the lower tract within a few days. Here, the virus infects the epithelial cells of the bronchial mucosa and pneumocytes in the alveoli. Viral replication initiates an influx of immune cells resulting in oedema, increased mucus secretion and sloughing of the infected cells leading to airway obstruction. Trapping of air distal to obstructed areas by means of the ball valve effect leads to atelectasis and VQ mismatch, leading to hypoxaemia. Regeneration of the bronchiolar epithelium begins within three to four days after the resolution of symptoms.

There are two major RSV subgroups – RSV A and RSV B. The subgroups can further be divided into several genotypes. These genotypes are based on reactions with monoclonal antibodies against the fusion (F) and attachment (G) glycoproteins. As of 2013, 11 RSV A and 17 RSV B genotypes have been identified. Group A and B co circulate within the community and predominance of one over the other depends on the year and geographical location. New genotypes continue to emerge while others become undetectable. Ongoing surveillance of these new and emerging genotypes have important implications in vaccine development. In a study done at Red Cross Children's Hospital in 2012, of all RSV identified, 80% were RSV A and 20% RSV B. The most prevalent genotypes included NA1, ON1 and NA2 – with NA1 the most predominant. Interestingly, type ON1 was first isolated in Canada in

2011. Isolates of the Canadian and South African ON1 strains are identical. It is unknown whether this demonstrates importation or natural evolution of the virus. The only circulating type B genotype isolated was BA4. There have been conflicting reports regarding the association of different genotypes with disease severity. Some studies report more severe disease with type A, while others type B.

RSV infection occurs in distinct seasonal patterns worldwide. In the Southern hemisphere, as demonstrated by data from the South African Severe Acute Respiratory Infections (SARIs) surveillance program, RSV circulates throughout the year with a peak between February and May. Outbreaks last an average of three to four months with peak incidence seen during the winter months. In South Africa the peak RSV season varies between provinces. Epidemics are usually seen in Kwazulu Natal in December/January, Gauteng in Feb/March and the Western Cape during March. This has important implications on timing of prophylactic interventions.

The WHO defines pneumonia as 'an acute episode of cough or difficulty breathing associated with an increased respiratory rate'. Severe pneumonia is described as 'the presence of lower chest wall indrawing requiring hospital admission' and is often characterized by hypoxia. RSV pneumonia typically begins with two to four days of low-grade fever, nasal congestion and rhinorrhea before presenting with lower respiratory tract symptoms of cough, tachypnea and increased respiratory effort (recessions, alar flare and grunting). In a study done in Pakistan, 85% of hospitalized children presented with cough followed by fever, nasal congestion and shortness of breath. Inspiratory crackles and expiratory wheeze were the most common findings on auscultation. When compared with other respiratory viruses in a large cohort of children in Paarl, RSV was associated with a higher prevalence of wheezing, lower chest wall indrawing and a higher median respiratory rate. Currently there is no scoring system available to predict whether illness will progress to severe disease.

Most children admitted to hospital with RSV pneumonia were previously healthy – 79% in one study. However, risk factors for severe disease have been identified. Chronologic age is the single most important predictor of the likelihood of severe disease. Two thirds of hospitalisations occur within the first five months of life, with almost half of those in the less than two-month age group. In a study done in Pakistan, it was shown that the risk of RSV linearly decreased with increasing age. The risk of being hospitalized with RSV pneumonia was 80% higher among the less than three-month age group than those older than a year. In a South African study done in a well vaccinated cohort of children between 2012 and 2014, the highest incidence of RSV pneumonia was found in the one to six-month age group with the most severe disease being seen at a median age of two months.

Most hospitalized infants are born full term with no known risk factors. However, there is an increased risk of severe disease and increased mortality in premature babies, with up to 13 times higher mortality in very low birth weight babies (<1500g) compared to babies born > 2500g. This can partly be explained by the lack of maternal immunoglobulins in premature infants with most maternal immunoglobulin transfer occurring in the third trimester. This is further supported by the high rates of hospitalization of infants between 30 and 90 days after birth – corresponding to the declining concentration of transplacentally acquired maternal immunoglobulin.

Preexisting cardiopulmonary disease has also been found to predispose to severe RSV disease. Risk of mortality from RSV has been found to increase three-fold among children with haemodynamically significant congenital heart defects. However, this data is old and may not reflect recent advances in corrective cardiac surgery. Chronic lung disease, including chronic lung disease of prematurity has also been found to be a risk factor for severe RSV disease.

Malnutrition and HIV are important risk factors for severe disease, especially relevant in LMICs such as South Africa. In the Global Burden of Disease Study in 2016, it was found that childhood wasting remained the leading risk factor for pneumonia mortality in children younger than five years – a factor contributing to 57,1% of pneumonia deaths in 2016. This association is thought to be related to the impaired immunity associated with malnutrition. It was calculated that for every 1576 children who improved their weight for height Z score, one death could have been prevented.

HIV infection has been associated with a two to three-fold increase in severe RSV disease, with higher case fatality. Some data report up to a 30 times higher mortality rate in children living with HIV. A South African study during 2010-2011 demonstrated a three to five-fold increased risk of hospitalization in HIV infected children aged less than one year. This increased risk remained higher for HIV infected children up until age five. It also demonstrated a poorer outcome in terms of length of hospital stay and death. Another national South African surveillance study found that HIV infected children with RSV LRTI had a 10 times greater risk of death compared to HIV uninfected children. One possible explanation for this is impairment of cell mediated immunity in HIV infected children needed for protection against RSV.

Other risk factors associated with severe disease include male gender, low socioeconomic status, household pollution and overcrowding, having siblings, maternal smoking, history of atopy and not being breastfed.

Identifying the specific causative pathogens in childhood pneumonia has many challenges. Upper respiratory tract specimens have become the most common specimen type used in diagnosing viral pneumonia due to ease of collection. Specimens from the lower respiratory tract are difficult to obtain, as children are usually unable to produce sputum. Many pathogens require sophisticated laboratory culture systems for growth or replication. Conventional diagnosis through cell culture or immunofluorescence has poor sensitivity. With the introduction of more sensitive nucleic acid detection tests, such as PCR our ability to identify pathogens has increased dramatically – compared with conventional methods, pathogen detection increases by about three fold.

Recent studies have shown that identifying a pathogen in a respiratory specimen does not necessarily indicate a causal relationship. Organisms once thought to have been pathogenic have now been found in otherwise healthy controls. However, there is evidence that the detection of RSV in respiratory samples is strongly associated with severe LRTI and need for hospitalization.

RSV disease is a variable but usually self-limiting illness with therapy mainly supportive. The use of nebulized therapy – both bronchodilator and hypertonic saline – has limited evidence of effectiveness in RSV LRTI. Oxygen therapy via high flow nasal cannula, CPAP or invasive ventilation may be needed if further respiratory support is required.

In 2015, the WHO's Product Development for Vaccines Advisory Committee identified RSV as 'a pathogen for which there is major vaccine pipeline activity' with a vaccine likely to be available in the next five to 10 years. As of 2017, about 50 RSV vaccines are currently in development. Two broad approaches to RSV immunization are being considered in children – maternal immunization for infants less than six months old and paediatric vaccines for children older than six months. It has been demonstrated that more severe disease is observed among infants born early in the RSV season, due to waning maternal immunity. These observations raise the possibility that maternal vaccination against RSV could have beneficial effects on the infant.

RATIONALE

Hypothesis

We hypothesise that RSV infection is involved in a substantial number of cases of severe lower respiratory tract infection in children requiring hospitalisation.

OBJECTIVES

1. To determine the proportion of infants and children admitted to Red Cross War Memorial Children's Hospital during one calendar year with acute lower respiratory tract infection, who are infected with RSV.
2. To determine the impact of HIV infection on the epidemiology, clinical presentation and outcome of RSV in children.
3. To determine the possible association of RSV infection with age, HIV status, nutritional status, tobacco and bio fuel exposure, immunization status and associated underlying chronic conditions.

METHODS

This study will use data that was prospectively collected over a one year period between September 2012 and September 2013 in the admission ward (S11) of the Red Cross War Memorial Children's Hospital in Cape Town South Africa.

Inclusion criteria

Infants and children aged less than 13 years who were admitted during the study period for lower respiratory tract infection as defined by WHO. Written, informed consent for enrolment was obtained from parent/ legal guardian.

Exclusion criteria

Any child who was deemed too ill to be enrolled in the study by the attending paediatrician was excluded. Children who had been admitted to the ward for a period of longer than 72 hours prior to recruitment were also excluded from the study.

A brief description of procedures undertaken to collect data

After obtaining consent, a review of each child's clinical notes was done and additional history taken from the primary caregiver. A physical examination was performed on each child and oxygen saturations measured and recorded. A chest X-ray was taken if clinically necessary and reported on by two clinicians trained on the WHO method of reporting chest radiographs for pneumonia in children. Both clinicians were blinded to the diagnosis. Where there was no agreement, a third clinician was asked to review the radiographs. From each child, a nasopharyngeal aspirate and induced sputum specimen was taken for molecular diagnostic testing.

HIV testing was performed on every child whose HIV status was unknown and performed after pretest counselling with informed consent from a parent or legal guardian. This consisted of an HIV ELISA test, plus a confirmatory PCR test if the child was 18 months or less or two confirmatory ELISA tests if the child was > 18 months old. Children were classified as ‘HIV infected’, ‘HIV uninfected exposed’ or; HIV uninfected unexposed’.

For the purpose of this study, nutritional status will be defined according to WHO criteria using their 2007 Reference Growth chars as follows:

- Moderate underweight for age: weight-for-age Z-score (WAZ) between -2 and -3
- Severe underweight for age: WAZ less than -3
- Moderate malnutrition: weight-for-height Z-score (WHZ) or height-for-age Z-score (HAZ) between -2 and -3
- Severe malnutrition: WHZ less than -3, visible wasting, or the presence of nutritional oedema

Management of study participants

All participants were managed according to the national and departmental guidelines, at the discretion of the attending paediatrician. Children were followed up until discharge and in-hospital course, duration and outcome were recorded. For children admitted to the paediatric ICU, the duration of IPPV, ventilation and oxygenation requirements were recorded. For children requiring oxygen, duration of oxygen therapy was recorded.

Sample size

The study involves a convenient sample based on already collected data. The database contains 460 participants. Previous studies such as the Drakenstein study, which was undertaken around the same time, showed the prevalence of RSV around 20%, which for our sample will give a 95% confidence interval (CI) of 16.3 % to 23.7%. The study will be able to give a precision within 5% above and below all prevalence point estimates from 2.5% to 50%. Table 1.

Table: 1 95% confidence interval around proportion of cases testing positive for RSV on PCR

Number enrolled	Proportion of RSV positive cases with upper and lower 95% confidence levels						
	2.5%	5%	10%	20%	30%	40%	50%
250	0.6; 4.4	2.3; 7.7	6.3; 13.7	15.0; 25.0	24.3; 35.7	33.9; 46.1	43.8; 56.2
350	0.9; 4.1	2.7; 7.3	6.9;13.1	15.8; 24.2	25.2-34.8	34.9-45.1	44.8-55.2
400	1.0; 4.0	2.9; 7.1	7.1; 12.9	16.1; 23.9	25.5; 34.5	35.2; 44.8	45.1; 54.9
450	1.1; 3.9	3.0; 7.0	7.2; 12.8	16.3; 23.7	25.8-34.2	35.5-44.5	45.4-54.6

Data management

The database currently resides in an access-controlled computer belonging to the principal investigator. Alphanumeric codes have been used to identify patients in order to protect their anonymity. Only the principal investigator has access to the patient's identity. A copy of the database has already been imported into STATA (StataCorp LP, College Station, TX), the program that will be used to analyse the data.

Statistical analysis

Demographic characteristics will be tabulated to provide a background description of the study population. Percentages and their 95 confidence intervals will be used to depict proportions of categorical variables while medians with interquartile ranges will be used to summarise all continuous variables, as appropriate. The χ^2 test or Fisher's exact test will be used to assess the strength of association between two categorical variables as appropriate. Continuous variables will be tested for normality and the appropriate statistical test used to test for association.

Factors that are associated with a positive PCR for RSV will be identified on a univariate analysis and any variables with $p<0.1$ will be further analysed by multivariable analysis. Some of the variables that will be assessed include age of the patient, HIV status and nutritional status. In the final multivariable model, risk ratios with 95 confidence intervals will be used to quantify the measure of effect between the RSV negative and RSV positive groups, controlled for relevant confounders.

A significance level of $P<0.05$ will be used for all analysis.

Risks and benefits

As the study uses data already collected, patient care will not be affected. We hope that quantifying of the RSV disease burden as well as identification of potential risk factors will improve the future care of children with severe LRTI.

Dissemination of study results

The findings of the study will be disseminated using various platforms such as in-house academic meetings and research days. In addition, we intend to submit the resultant manuscript for peer review for possible publication in an appropriate peer reviewed journal.

Ethical considerations

The parent study has already received ethics approval (HREC 371/2011), however approval for the current analysis will be sought from the Departmental Research Committee of the Department of Paediatrics & Child Health, after which an expedited review will be requested from the Human Research Ethics Committee of the University of Cape Town. The investigator will ensure that this study is conducted in full conformity with the principles set forth in the research guideline for Good Clinical Practice and the Declaration of Helsinki in its current version, whichever affords the greater protection to the participants.

Limitations of study

As the data being used has already been collected, there is a possibility that there may be missing data that specifically pertains to RSV disease, that ideally should have been collected. However, we are confident that the initial study was exhaustive with the collection of data pertaining to severe respiratory illness.

Budget

All logistics for the acquisition of data for this study have already been covered by the parent study. We however do envisage a need for statistical support as well as cost associated with open source publication. The estimated costs are therefore:

- Student license for Stata program for data analysis – R 3 700
- Fees for statistical consultation – R 2000
- *Publication fees – R 6 800 to R 20 000, depending on choice of journal

(* The supervisor has indicated that he will look for other sources of funding to offset these costs)

Operational Plan/Timelines

The project is expected to be completed over a six-month period as per the timetable set in Table 2.

Table 2. Operational Plan/Timelines

	Sep 2019	Oct 2019	Nov 2019	Dec 2019	Jan 2020	Feb 2020
Submit protocol	X					
Data clean-up		X				
Analysis and initial draft			X	X		
Final Report					X	X

Reference section removed.

Appendix Two: Ethical approval

UNIVERSITY OF CAPE TOWN
Faculty of Health Sciences
Human Research Ethics Committee

Room E53-46 Old Main Building
Groote Schuur Hospital
Observatory 7925
Telephone [021] 406 6626
Email: Olivia.langenhoven@uct.ac.za
Website: www.health.uct.ac.za/fhs/research/humanethics/forms

24 October 2019

HREC REF:726/2019

A/Prof Rudzani Muloiwa
Division of Paediatrics & Child Health
G26 NMB GSH

Dear A/Prof Muloiwa

PROJECT TITLE: RSV INFECTION IN CHILDREN HOSPITALISED WITH SEVERE LOWER RESPIRATORY TRACT INFECTION AT THE RED CROSS WAR MEMORIAL CHILDREN'S HOSPITAL (2012-2013) (SUB-STUDY to 371/2011) (MMED in paediatrics candidate Dr N Morgan)

Thank you for submitting your study to the Faculty of Health Sciences Human Research Ethics Committee.

It is a pleasure to inform you that the HREC has **formally approved** the above-mentioned study.

Approval is granted for one year until the 30 October 2020.

Please submit a progress form, using the standardised Annual Report Form if the study continues beyond the approval period. Please submit a Standard Closure form if the study is completed within the approval period.
(Forms can be found on our website: www.health.uct.ac.za/fhs/research/humanethics/forms)

Please note that for all studies approved by the HREC, the principal investigator **must** obtain appropriate institutional approval, where necessary, before the research may occur.

Please note that the ongoing ethical conduct of the study remains the responsibility of the principal investigator.

We acknowledge that the student: Dr N. Morgan will also be involved in this study.

Please quote the HREC REF in all your correspondence.

Yours sincerely

PROFESSOR M BLOCKMAN
CHAIRPERSON, FHS HUMAN RESEARCH ETHICS COMMITTEE
Federal Wide Assurance Number: FWA00001637.
Institutional Review Board (IRB) number: IRB00001938

HREC REF 726/2019

Appendix Three: PLOS ONE Author Instructions

Style and Format

File format

Manuscript files can be in the following formats: DOC, DOCX, or RTF. Microsoft Word documents should not be locked or protected.

LaTeX manuscripts must be submitted as PDFs. Read the LaTeX guidelines.

Length

Manuscripts can be any length. There are no restrictions on word count, number of figures, or amount of supporting information.

We encourage you to present and discuss your findings concisely.

Font

Use a standard font size and any standard font, except for the font named “Symbol”. To add symbols to the manuscript, use the Insert → Symbol function in your word processor or paste in the appropriate Unicode character.

Headings

Limit manuscript sections and sub-sections to 3 heading levels. Make sure heading levels are clearly indicated in the manuscript text.

Layout and spacing

Manuscript text should be double-spaced.

Do not format text in multiple columns.

Page and line numbers

Include page numbers and line numbers in the manuscript file. Use continuous line numbers (do not restart the numbering on each page).

Footnotes

Footnotes are not permitted. If your manuscript contains footnotes, move the information into the main text or the reference list, depending on the content.

Language

Manuscripts must be submitted in English.

You may submit translations of the manuscript or abstract as supporting information. Read the supporting information guidelines.

Abbreviations

Define abbreviations upon first appearance in the text.

Do not use non-standard abbreviations unless they appear at least three times in the text.

Keep abbreviations to a minimum.

Reference style

PLOS uses “Vancouver” style, as outlined in the ICMJE sample references.

See reference formatting examples and additional instructions below.

Equations

We recommend using MathType for display and inline equations, as it will provide the most reliable outcome. If this is not possible, Equation Editor or Microsoft's Insert→Equation function is acceptable.

Avoid using MathType, Equation Editor, or the Insert→Equation function to insert single variables (e.g., “$a^2 + b^2 = c^2$”), Greek or other symbols (e.g., β, Δ, or ′ [prime]), or mathematical operators (e.g., x, ≥, or ±) in running text. Wherever possible, insert single symbols as normal text with the correct Unicode (hex) values.

Do not use MathType, Equation Editor, or the Insert→Equation function for only a portion of an equation. Rather, ensure that the entire equation is included. Equations should not contain a mix of different equation tools. Avoid “hybrid” inline or display equations, in which part is text and part is MathType, or part is MathType and part is Equation Editor.

Nomenclature

Use correct and established nomenclature wherever possible.

Units of measurement Use SI units. If you do not use these exclusively, provide the SI value in parentheses after each value. Read more about SI units.

Drugs Provide the Recommended International Non-Proprietary Name (rINN).

Species names Write in italics (e.g., Homo sapiens). Write out in full the genus and species, both in the title of the manuscript and at the first mention of an organism in a paper. After first mention, the first letter of the genus name followed by the full species name may be used (e.g., H. sapiens).

Genes, mutations, genotypes, and alleles Write in italics. Use the recommended name by consulting the appropriate genetic nomenclature database (e.g., HGNC for human genes; we strongly recommend using this tool to check against previously approved names). It is sometimes advisable to indicate the synonyms for the gene the first time it appears in the text. Gene prefixes such as those used for oncogenes or cellular localization should be shown in roman typeface (e.g., v-fes, c-MYC).

Allergens

The systematic allergen nomenclature of the World Health Organization/International Union of Immunological Societies (WHO/IUIS) Allergen Nomenclature Sub-committee should be used for manuscripts that include the description or use of allergenic proteins. For manuscripts describing new allergens, the systematic name of the allergen should be approved by the WHO/IUIS Allergen Nomenclature Sub-Committee prior to manuscript publication. Examples of the systematic allergen nomenclature can be found at the WHO/IUIS Allergen Nomenclature site.

Copyediting manuscripts

Prior to submission, authors who believe their manuscripts would benefit from professional editing are encouraged to use language-editing and copyediting services. Obtaining this service is the responsibility of the author, and should be done before initial submission. These services can be found on the web using search terms like “scientific editing service” or “manuscript editing service.”

Submissions are not copyedited before publication.

Submissions that do not meet the PLOS ONE publication criterion for language standards may be rejected.

Manuscript Organization

Manuscripts should be organized as follows. Instructions for each element appear below the list.

Beginning section

The following elements are required, in order:

Title page: List title, authors, and affiliations as first page of the manuscript

Abstract

Introduction

Middle section

The following elements can be renamed as needed and presented in any order:

Materials and Methods

Results

Discussion

Conclusions (optional)

Ending section

The following elements are required, in order:

Acknowledgments

References

Supporting information captions (if applicable)

Other elements

Figure captions are inserted immediately after the first paragraph in which the figure is cited. Figure files are uploaded separately.

Tables are inserted immediately after the first paragraph in which they are cited.

Supporting information files are uploaded separately.

Refer to our downloadable sample files to ensure that your submission meets our formatting requirements:

Download sample title, author list, and affiliations page (PDF)

Download sample manuscript body (PDF)

Viewing Figures and Supporting Information in the compiled submission PDF

The compiled submission PDF includes low-resolution preview images of the figures after the reference list. The function of these previews is to allow you to download the entire submission as quickly as possible. Click the link at the top of each preview page to download a high-resolution version of each figure. Links to download Supporting Information files are also available after the reference list.

Parts of a Submission

Title

Include a full title and a short title for the manuscript.

Title Length Guidelines Examples

Full title 250 charactersSpecific, descriptive, concise, and comprehensible to readers outside the field

Impact of cigarette smoke exposure on innate immunity: A Caenorhabditis elegans model

Solar drinking water disinfection (SODIS) to reduce childhood diarrhoea in rural Bolivia: A cluster-randomized, controlled trial

Short title 100 charactersState the topic of the study

Cigarette smoke exposure and innate immunity

SODIS and childhood diarrhoea

Titles should be written in sentence case (only the first word of the text, proper nouns, and genus names are capitalized). Avoid specialist abbreviations if possible. For clinical trials, systematic reviews, or meta-analyses, the subtitle should include the study design.

Author list

Authorship requirements

All authors must meet the criteria for authorship as outlined in the authorship policy. Those who contributed to the work but do not meet the criteria for authorship can be mentioned in the Acknowledgments. Read more about Acknowledgments.

The corresponding author must provide an ORCID iD at the time of submission by entering it in the user profile in the submission system. Read more about ORCID.

Author names and affiliations

Enter author names on the title page of the manuscript and in the online submission system.

On the title page, write author names in the following order:

First name (or initials, if used)

Middle name (or initials, if used)

Last name (surname, family name)

Each author on the list must have an affiliation. The affiliation includes department, university, or organizational affiliation and its location, including city, state/province (if applicable), and country. Authors have the option to include a current address in addition to the address of their affiliation at the time of the study. The current address should be listed in the byline and clearly labeled “current address.” At a minimum, the address must include the author’s current institution, city, and country.

If an author has multiple affiliations, enter all affiliations on the title page only. In the submission system, enter only the preferred or primary affiliation. Author affiliations will be listed in the typeset PDF article in the same order that authors are listed in the submission.

Author names will be published exactly as they appear in the manuscript file. Please double-check the information carefully to make sure it is correct.

Corresponding author

The submitting author is automatically designated as the corresponding author in the submission system. The corresponding author is the primary contact for the journal office and the only author able to view or change the manuscript while it is under editorial consideration.

The corresponding author role may be transferred to another coauthor. However, note that transferring the corresponding author role also transfers access to the manuscript. (To designate a new corresponding author while the manuscript is still under consideration, watch the video tutorial below.)

Only one corresponding author can be designated in the submission system, but this does not restrict the number of corresponding authors that may be listed on the article in the event of publication. Whoever is designated as a corresponding author on the title page of the manuscript file will be listed as such upon publication. Include an email address for each corresponding author listed on the title page of the manuscript.

How to select a new corresponding author in Editorial Manager

Consortia and group authorship

If a manuscript is submitted on behalf of a consortium or group, include its name in the manuscript byline. Do not add it to the author list in the submission system. You may include the full list of members in the Acknowledgments or in a supporting information file.

PubMed only indexes individual consortium or group author members listed in the article byline. If included, these individuals must qualify for authorship according to our criteria.

Read the group authorship policy.

Author contributions

Provide at minimum one contribution for each author in the submission system. Use the CRediT taxonomy to describe each contribution. Read the policy and the full list of roles.

Contributions will be published with the final article, and they should accurately reflect contributions to the work. The submitting author is responsible for completing this information at submission, and we expect that all authors will have reviewed, discussed, and agreed to their individual contributions ahead of this time. PLOS ONE will contact all authors by email at submission to ensure that they are aware of the submission.

Cover letter

Upload a cover letter as a separate file in the online system. The length limit is 1 page.

The cover letter should include the following information:

Summarize the study’s contribution to the scientific literature

Relate the study to previously published work

Specify the type of article (for example, research article, systematic review, meta-analysis, clinical trial)

Describe any prior interactions with PLOS regarding the submitted manuscript

Suggest appropriate Academic Editors to handle your manuscript (see the full list of Academic Editors)

List any opposed reviewers

IMPORTANT: Do not include requests to reduce or waive publication fees in the cover letter. This information will be entered separately in the online submission system.

Read about publication fee assistance.

Title page

The title, authors, and affiliations should all be included on a title page as the first page of the manuscript file.

Download our sample title, author list, and affiliations page (PDF)

Abstract

The Abstract comes after the title page in the manuscript file. The abstract text is also entered in a separate field in the submission system.

The Abstract should:

Describe the main objective(s) of the study

Explain how the study was done, including any model organisms used, without methodological detail

Summarize the most important results and their significance

Not exceed 300 words

Abstracts should not include:

Citations

Abbreviations, if possible

Introduction

The introduction should:

Provide background that puts the manuscript into context and allows readers outside the field to understand the purpose and significance of the study

Define the problem addressed and why it is important

Include a brief review of the key literature

Note any relevant controversies or disagreements in the field

Conclude with a brief statement of the overall aim of the work and a comment about whether that aim was achieved

Materials and Methods

The Materials and Methods section should provide enough detail to allow suitably skilled investigators to fully replicate your study. Specific information and/or protocols for new methods should be included in detail. If materials, methods, and protocols are well established, authors may cite articles where those protocols are described in detail, but the submission should include sufficient information to be understood independent of these references.

Supporting reproducibility with protocols

To enhance the reproducibility of your results, we recommend and encourage you to make your protocols public. There are several options:

Protocols associated with Research Articles

Protocol documents may be uploaded as Supporting Information or linked from the Methods section of the article. For laboratory protocols, we recommend protocols.io. Include the DOI link in the Methods section of your manuscript using the following format: http://dx.doi.org/10.17504/protocols.io.[PROTOCOL DOI]. This allows editors and reviewers to consult the detailed step-by-step protocol when evaluating your manuscript. You can choose to keep the protocol private on the protocols.io platform until your article is published—at which time it will be published automatically.

Protocols published in their own right

PLOS ONE offers two options for publishing stand-alone protocol articles: Lab Protocols that describe reusable methodologies and Study Protocols that describe detailed plans and proposals for research projects. Specific guidelines apply to the submission of Lab Protocol and Study Protocol manuscripts. Read the detailed instructions for submitting Lab Protocols and Study Protocols.

Results, Discussion, Conclusions

These sections may all be separate, or may be combined to create a mixed Results/Discussion section (commonly labeled "Results and Discussion") or a mixed Discussion/Conclusions section (commonly labeled "Discussion"). These sections may be further divided into subsections, each with a concise subheading, as appropriate. These sections have no word limit, but the language should be clear and concise.

Together, these sections should describe the results of the experiments, the interpretation of these results, and the conclusions that can be drawn.

Authors should explain how the results relate to the hypothesis presented as the basis of the study and provide a succinct explanation of the implications of the findings, particularly in relation to previous related studies and potential future directions for research.

PLOS ONE editorial decisions do not rely on perceived significance or impact, so authors should avoid overstating their conclusions. See the PLOS ONE Criteria for Publication for more information.

Acknowledgments

Those who contributed to the work but do not meet our authorship criteria should be listed in the Acknowledgments with a description of the contribution.

Authors are responsible for ensuring that anyone named in the Acknowledgments agrees to be named.

PLOS journals publicly acknowledge the indispensable efforts of our editors and reviewers on an annual basis. To ensure equitable recognition and avoid any appearance of partiality, do not include editors or peer reviewers—named or unnamed—in the Acknowledgments.

Do not include funding sources in the Acknowledgments or anywhere else in the manuscript file. Funding information should only be entered in the financial disclosure section of the submission system.

References

Any and all available works can be cited in the reference list. Acceptable sources include:

Published or accepted manuscripts

Manuscripts on preprint servers, providing the manuscript has a citable DOI or arXiv URL.

Do not cite the following sources in the reference list:

Unavailable and unpublished work, including manuscripts that have been submitted but not yet accepted (e.g., “unpublished work,” “data not shown”). Instead, include those data as supplementary material or deposit the data in a publicly available database.

Personal communications (these should be supported by a letter from the relevant authors but not included in the reference list)

Submitted research should not rely upon retracted research. You should avoid citing retracted articles unless you need to discuss retracted work to provide historical context for your submitted research. If it is necessary to discuss retracted work, state the article's retracted status in your article's text and reference list.

Ensure that your reference list includes full and current bibliography details for every cited work at the time of your article's submission (and publication, if accepted). If cited work is corrected, retracted, or marked with an expression of concern before your article is published, and if you feel it is appropriate to cite the work even in light of the post-publication notice, include in your manuscript citations and full references for both the affected article and the post-publication notice. Email the journal office if you have questions.

References are listed at the end of the manuscript and numbered in the order that they appear in the text. In the text, cite the reference number in square brackets (e.g., "We used the techniques developed by our colleagues [19] to analyze the data"). PLOS uses the numbered citation (citation-sequence) method and first six authors, et al.

Do not include citations in abstracts.

Make sure the parts of the manuscript are in the correct order before ordering the citations.

Formatting references

Because all references will be linked electronically as much as possible to the papers they cite, proper formatting of references is crucial.

PLOS uses the reference style outlined by the International Committee of Medical Journal Editors (ICMJE), also referred to as the "Vancouver" style. Example formats are listed below. Additional examples are in the ICMJE sample references.

A reference management tool, EndNote, offers a current style file that can assist you with the formatting of your references. If you have problems with any reference management program, please contact the source company's technical support. Journal name abbreviations should be those found in the National Center for Biotechnology Information (NCBI) databases.

Source Format

Published articles

Hou WR, Hou YL, Wu GF, Song Y, Su XL, Sun B, et al. cDNA, genomic sequence cloning and overexpression of ribosomal protein gene L9 (rpL9) of the giant panda (Ailuropoda melanoleuca). Genet Mol Res. 2011;10: 1576-1588.

Devaraju P, Gulati R, Antony PT, Mithun CB, Negi VS. Susceptibility to SLE in South Indian Tamils may be influenced by genetic selection pressure on TLR2 and TLR9 genes. Mol Immunol. 2014 Nov 22. pii: S0161-5890(14)00313-7. doi: 10.1016/j.molimm.2014.11.005.

Note: A DOI number for the full-text article is acceptable as an alternative to or in addition to traditional volume and page numbers. When providing a DOI, adhere to the format in the example above with both the label and full DOI included at the end of the reference (doi: 10.1016/j.molimm.2014.11.005). Do not provide a shortened DOI or the URL.

Accepted, unpublished articles Same as published articles, but substitute “Forthcoming” for page numbers or DOI.

Online articles

Huynen MMTE, Martens P, Hilderlink HBM. The health impacts of globalisation: a conceptual framework. Global Health. 2005;1: 14. Available from: http://www.globalizationandhealth.com/content/1/1/14

Books

Bates B. Bargaining for life: A social history of tuberculosis. 1st ed. Philadelphia: University of Pennsylvania Press; 1992.

Book chapters Hansen B. New York City epidemics and history for the public. In: Harden VA, Risse GB, editors. AIDS and the historian. Bethesda: National Institutes of Health; 1991. pp. 21-28.

Deposited articles (preprints, e-prints, or arXiv)

Krick T, Shub DA, Verstraete N, Ferreiro DU, Alonso LG, Shub M, et al. Amino acid metabolism conflicts with protein diversity. arXiv:1403.3301v1 [Preprint]. 2014 [cited 2014 March 17]. Available from: https://128.84.21.199/abs/1403.3301v1

Kording KP, Mensh B. Ten simple rules for structuring papers. BioRxiv [Preprint]. 2016 bioRxiv 088278 [posted 2016 Nov 28; revised 2016 Dec 14; revised 2016 Dec 15; cited 2017 Feb 9]: [12 p.]. Available from: https://www.biorxiv.org/content/10.1101/088278v5 doi: 10.1101/088278

Published media (print or online newspapers and magazine articles) Fountain H. For Already Vulnerable Penguins, Study Finds Climate Change Is Another Danger. The New York Times. 2014 Jan 29 [Cited 2014 March 17]. Available from: http://www.nytimes.com/2014/01/30/science/earth/climate-change-taking-toll-on-penguins-study-finds.html

New media (blogs, web sites, or other written works) Allen L. Announcing PLOS Blogs. 2010 Sep 1 [cited 17 March 2014]. In: PLOS Blogs [Internet]. San Francisco: PLOS 2006 - . [about 2 screens]. Available from: http://blogs.plos.org/plos/2010/09/announcing-plos-blogs/.

Masters' theses or doctoral dissertations Wells A. Exploring the development of the independent, electronic, scholarly journal. M.Sc. Thesis, The University of Sheffield. 1999. Available from: http://cumincad.scix.net/cgi-bin/works/Show?2e09

Databases and repositories (Figshare, arXiv) Roberts SB. QPX Genome Browser Feature Tracks; 2013 [cited 2013 Oct 5]. Database: figshare [Internet]. Available from: http://figshare.com/articles/QPX_Genome_Browser_Feature_Tracks/701214

Multimedia (videos, movies, or TV shows) Hitchcock A, producer and director. Rear Window [Film]; 1954. Los Angeles: MGM.

Supporting information

Authors can submit essential supporting files and multimedia files along with their manuscripts. All supporting information will be subject to peer review. All file types can be submitted, but files must be smaller than 20 MB in size.

Authors may use almost any description as the item name for a supporting information file as long as it contains an “S” and number. For example, “S1 Appendix” and “S2 Appendix,” “S1 Table” and “S2 Table,” and so forth.

Supporting information files are published exactly as provided, and are not copyedited.

Supporting information captions

List supporting information captions at the end of the manuscript file. Do not submit captions in a separate file.

The file number and name are required in a caption, and we highly recommend including a one-line title as well. You may also include a legend in your caption, but it is not required.

Example caption

S1 Text. Title is strongly recommended. Legend is optional.

In-text citations

We recommend that you cite supporting information in the manuscript text, but this is not a requirement. If you cite supporting information in the text, citations do not need to be in numerical order.

Read the supporting information guidelines for more details about submitting supporting information and multimedia files.

Figures and tables

Figures

Do not include figures in the main manuscript file. Each figure must be prepared and submitted as an individual file.

Cite figures in ascending numeric order at first appearance in the manuscript file.

Read the guidelines for figures and requirements for reporting blot and gel results.

Figure captions

Figure captions must be inserted in the text of the manuscript, immediately following the paragraph in which the figure is first cited (read order). Do not include captions as part of the figure files themselves or submit them in a separate document.

At a minimum, include the following in your figure captions:

A figure label with Arabic numerals, and “Figure” abbreviated to “Fig” (e.g. Fig 1, Fig 2, Fig 3, etc). Match the label of your figure with the name of the file uploaded at submission (e.g. a figure citation of “Fig 1” must refer to a figure file named “Fig1.tif”).

A concise, descriptive title

The caption may also include a legend as needed.

Read more about figure captions.

Tables

Cite tables in ascending numeric order upon first appearance in the manuscript file.

Place each table in your manuscript file directly after the paragraph in which it is first cited (read order). Do not submit your tables in separate files.

Tables require a label (e.g., “Table 1”) and brief descriptive title to be placed above the table. Place legends, footnotes, and other text below the table.

Read the guidelines for tables.

Statistical reporting

Manuscripts submitted to PLOS ONE are expected to report statistical methods in sufficient detail for others to replicate the analysis performed. Ensure that results are rigorously reported in accordance with community standards and that statistical methods employed are appropriate for the study design.

Appendix Four: Turnitin Report

04/01/2022
This Turnitin Report has been verified and approved by co-supervisor A/Prof Heloise Buys

mrgnic001:Turnitin_version_M MED.docx

by Nicole Morgan

Submission date: 02-Jan-2023 09:10AM (UTC+0200)

Submission ID: 1987899889

File name: c572f609-8a02-4101-85a0-fb7e02fce24b_Turnitin_version_MMED.docx

(744.77K)

Word count: 3883

Character count: 20867