

Journal Pre-proof

Influenza-like illness is associated with high pneumococcal carriage density in Malawian children.

Tinashe K. Nyazika , Alice Law , Todd D. Swarthout ,
Lusako Sibale , Danielle ter Braake , Neil French ,
Robert S. Heyderman , Dean Everett , Aras Kadioglu ,
Kondwani C. Jambo , Daniel R. Neill

PII: S0163-4453(20)30495-3
DOI: <https://doi.org/10.1016/j.jinf.2020.06.079>
Reference: YJINF 4761



To appear in: *Journal of Infection*

Accepted date: 27 June 2020

Please cite this article as: Tinashe K. Nyazika , Alice Law , Todd D. Swarthout , Lusako Sibale , Danielle ter Braake , Neil French , Robert S. Heyderman , Dean Everett , Aras Kadioglu , Kondwani C. Jambo , Daniel R. Neill , Influenza-like illness is associated with high pneumococcal carriage density in Malawian children., *Journal of Infection* (2020), doi: <https://doi.org/10.1016/j.jinf.2020.06.079>

This is a PDF file of an article that has undergone enhancements after acceptance, such as the addition of a cover page and metadata, and formatting for readability, but it is not yet the definitive version of record. This version will undergo additional copyediting, typesetting and review before it is published in its final form, but we are providing this version to give early visibility of the article. Please note that, during the production process, errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

© 2020 Published by Elsevier Ltd on behalf of The British Infection Association.

Influenza-like illness is associated with high pneumococcal carriage density in Malawian children.

Running Title: Pneumococcal carriage density during ILI

Tinashe K. Nyazika ^{a,b,c1*}, Alice Law ^{d1}, Todd D. Swarthout ^{a,e}, Lusako Sibale ^a, Danielle ter Braake ^{d,f}, Neil French ^d, Robert S. Heyderman ^e, Dean Everett ^g, Aras Kadioglu ^{d2}, Kondwani C. Jambo ^{a,b2*}, Daniel R. Neill ^{d2}

^a Malawi-Liverpool-Wellcome Trust Clinical Research Programme, University of Malawi College of Medicine, Blantyre, Malawi

^b Department of Clinical Sciences, Liverpool School of Tropical Medicine, Liverpool, United Kingdom

^c Department of Pathology, College of Health Sciences, University of Malawi, Blantyre, Malawi

^d Institute of Infection and Global Health, University of Liverpool, Liverpool, United Kingdom

^e NIHR Global Health Research Unit on Mucosal Pathogens, Division of Infection and Immunity, University College London, London, United Kingdom

^f Department of Biomolecular Health Sciences, Division of Infectious Diseases & Immunology, Faculty of Veterinary Medicine, Utrecht, The Netherlands

^g The Queens Medical Research Institute, University of Edinburgh, Edinburgh, United Kingdom.

¹ TKN and AL contributed equally to this work

² AK, KCJ and DRN contributed equally to this work

Corresponding authors

Email: tknyazika@mlw.mw (TKN); Email: kjambo@mlw.mw (KCJ)

Highlights

- Influenza-like illness (ILI) in children is associated with high pneumococcal carriage density.
- Children with ILI harboured more viral organisms than asymptomatic healthy children.
- Children with ILI patients had higher IL-8 levels in nasal aspirates than asymptomatic healthy children

Journal Pre-proof

SUMMARY

Background: High pneumococcal carriage density is a risk factor for invasive pneumococcal disease (IPD) and transmission, but factors that increase pneumococcal carriage density are still unclear.

Methods: We undertook a cross-sectional study to evaluate the microbial composition, cytokine levels and pneumococcal carriage densities in samples from children presenting with an influenza-like illness (ILI) and asymptomatic healthy controls (HC).

Results: The proportion of children harbouring viral organisms (Relative risk (RR) 1.4, $p=0.0222$) or ≥ 4 microbes at a time (RR 1.9, $p<0.0001$), was higher in ILI patients than HC. ILI patients had higher IL-8 levels in nasal aspirates than HC (median [IQR], 265.7 [0 – 452.3] vs. 0 [0 – 127.3] pg/ml; $p = 0.0154$). Having an ILI was associated with higher pneumococcal carriage densities compared to HC (RR 4.2, $p<0.0001$).

Conclusion: These findings suggest that children with an ILI have an increased propensity for high pneumococcal carriage density. This could in part contribute to increased susceptibility to IPD and transmission in the community.

Word count: 160 words

Keywords: Influenza-like illness, carriage density, pneumococcal, IL-8, Children

Introduction

Pneumococcal carriage is a prerequisite for disease and an important step for transmission. In Malawi, pneumococcus is known to be the major cause of meningitis and second cause of bacteraemia and community acquired pneumonia¹⁻⁵. The majority of invasive pneumococcal disease (IPD) cases are seen in children under the ages of 5 and carriage rates are known to increase with age between 0 to 5yrs⁶⁻⁸. IPD cases have been shown to be on the decrease in Africa, including Malawi with uptake of the PCV^{9,10}. Pneumococcal meningitis frequently seen in those between the ages of 6 –18 months, while bacteraemia is commonly seen amongst those aged 6 – 36 months and pneumococcal pneumonia occurring in children between 3 – 60 months of age^{9,11}. In Malawi, pneumococcal disease peaks during the colder and drier months, with serotypes 1, 6A/B, 14 and 23F being major causes of IPD in children^{1,5,12-14}.

Pneumococcal carriage is common in children, with approximately 80% of under-fives in Malawi carrying at a given time¹⁵, and is influenced by the microbial composition of the upper respiratory tract (URT), including viral co-infections¹⁶⁻²¹. Colonisation studies have demonstrated that microbe–microbe competition and synergy occur in the URT^{18,22,23}. For example, strong positive associations of pneumococcal nasopharyngeal carriage with rhinovirus, influenza virus, respiratory syncytial virus (RSV) and parainfluenza virus have been reported²⁴⁻²⁶.

Viral-induced local inflammation in the nasal mucosa has been implicated as an important factor that promotes pneumococcal carriage and transmission²⁷⁻²⁹. Respiratory viral infections are associated with increased pneumococcal carriage density^{25,27,30-32}. Specifically, a direct correlation between heightened mucosal inflammation and high pneumococcal carriage density has been reported in children with RSV infection²⁷. Inflammation in the nasal mucosa drives epithelial denudation, up-regulation of platelet-

activating factor receptor (PAFr) and polymeric immunoglobulin receptor (pIgR) on epithelial cells, resulting in increased adherence of pneumococci and evasion of the nasal mucosal surface³³⁻³⁶. These observations highlight the important association, clinical and epidemiological, between virally induced inflammation in the URT and the pneumococcus.

Influenza-like illness (ILI) is prevalent amongst children³⁷⁻³⁹, with respiratory viral and bacterial pathogens reported as the key etiological agents^{40,41}. Recognising that ILI is common in Malawi and that the aetiology of ILI induces inflammation which increases both risk of carriage and carriage density, we hypothesised that children with an influenza-like illness (ILI) residing in Malawi, are more likely to harbour higher pneumococcal carriage densities than asymptomatic healthy controls. We conducted a cross-sectional study in children, recruiting those with and without ILI symptoms. Here, we show that children with an ILI harboured more respiratory microbes per individual, exhibited higher levels of IL-8 in the nasal mucosa, and had increased likelihood of high pneumococcal carriage densities, than asymptomatic healthy controls. These findings have potential broader implications in the development of interventions to curb pneumococcal disease and transmission.

Methods

Study design and participants

HIV-uninfected children were recruited into a comparative cross-sectional study from the Gateway clinic, a government primary healthcare facility and surrounding communities, between June and September 2017. All participants were from within Blantyre, a commercial city in the southern part of Malawi. Children between the ages of 1 and 10 years were conveniently sampled into two groups; children fulfilling the WHO influenza-like-illness (ILI) case definitions and community asymptomatic healthy controls. ILI was defined as having an acute respiratory illness (ARI) of recent onset (within 10 days of screening)

manifested by fever ($\geq 38.0^{\circ}\text{C}$), cough but not requiring hospitalisation⁴². We excluded participants that had received antibiotics at least 14-days prior to recruitment into the study. HIV status was confirmed using a single rapid test kit, STAT-VIEW HIV 1/2 assay (ChemBio Diagnostic Systems Inc, USA), and willingness to test was part of the inclusion criteria. Written informed consent were obtained from parents/guardians for children under the age of 8 and assents for children aged 8 and above. Ethical approval was obtained from the local ethics committee COMREC (P.07/16/1990) and University of Liverpool (Ref:0783).

Nasopharyngeal swab

A nylon nasal swab (FLOQSwabsTM, Copan Diagnostics, USA) was inserted into the nostril to the nasopharynx, a depth equal to the outer ear and left in place for 2-3 seconds before slow removal with a rotating movement. Two different swabs were used to sample both nares, using the same method. The swabs were placed in 1 ml skim milk-tryptone-glucose-glycerol (STGG) media and transported to the Malawi-Liverpool-Wellcome Trust laboratories for processing.

Nasal aspirates

Nasopharyngeal secretions were aspirated through a disposable sterile catheter connected to a mucus trap and vacuum source. The catheter was inserted into a nostril, directed posteriorly and toward the opening of the external ear to allow extraction from the nasopharynx. Suction (100-120mmHg for children; 120-150mmHg for adults) was applied whilst the catheter was slowly withdrawn using a rotating motion. Mucus from the other nostril was collected with the same catheter, using the same method. After mucus collection

from both nostrils, the catheter was flushed with 2 ml sterile phosphate buffered saline.

Mucus aspirates were vortexed, aliquoted and frozen at -80°C within 1-hour of collection.

***Streptococcus pneumoniae* culture and *lytA* PCR**

Nasopharyngeal (NP) swabs, after collection and prior to initial freezing, were vortexed and 100µl of the suspension cultured on sheep blood agar with gentamicin (SBG; 7% sheep blood agar, 5 µL gentamicin/mL) overnight at 37°C and 5% CO₂. Plates showing no *S. pneumoniae* growth were incubated for a further 24-hours before being reported as negative. *S. pneumoniae* was identified by colony morphology and optochin disc (Oxoid, Basingstoke, UK) susceptibility and bile solubility test was done on isolates with zone diameter <14mm. Nasopharyngeal pneumococcal carriage was detected via qPCR, targeting *S. pneumoniae* virulence gene *lytA* and semi-quantitative microbiological culture (Miles and Misra), which was positively correlated with regards to detection and density determination and has been shown previously⁴³. Samples were classified as positive for pneumococci by the presence of growth by culture and/or if *lytA* qPCR signals were >10 DNA copies, <40 cycles.

Multiplex real-time PCR detection of 33 respiratory microbes

Total nucleic acids were extracted from 300µl aliquots of the nasal aspirate by manual extraction using the QIAamp DNA Mini Kit (Qiagen, Manchester, UK), according to manufacturer's instructions. 10µl of total nucleic acid extracted was used for the Fast-track Diagnostics (FTD[®]; Luxembourg) multiplex. The multiplex real-time PCR uses the principle of the TaqMan[®] technology to detect pathogen genes. The kit detects 33 respiratory microbes namely, influenza A virus; influenza B virus; influenza C virus; influenza A(H1N1) virus (swine-lineage); human parainfluenza viruses 1, 2, 3 and 4; human coronaviruses NL63,

229E, OC43 and HKU1; human metapneumoviruses A/B; human rhinovirus; human respiratory syncytial viruses A/B; *human adenovirus*; *enterovirus*; *human parechovirus*; *human bocavirus*; *Pneumocystis jirovecii*; *Mycoplasma pneumoniae*; *Chlamydomphila pneumoniae*; *Streptococcus pneumoniae*; *Haemophilus influenzae B*; *Staphylococcus aureus*; *Moraxella catarrhalis*; *Bordetella spp.*; *Klebsiella pneumoniae*; *Legionella pneumophila/longbeachae*; *Salmonella spp.*; *Haemophilus influenzae*. All targeted microorganisms in a sample with a cycle threshold value of >10 and ≤ 37 were considered positive for that pathogen.

Cytokine measurements

IL-8, IL-10 and interferon (IFN)- γ levels in nasal aspirates were quantified using Quantikine ELISA kits (R&D systems, Minneapolis, USA), according to manufacturer instructions. Levels of active TGF- β within nasal aspirates was determined using luciferase-reporting transformed mink lung epithelial cells (MLEC) stably transfected with the expression construct p800neoLUC, containing a plasminogen activator inhibitor-1 (PAI-1) promoter fused to the firefly luciferase reporter gene. MLEC cells were cultured, and TGF- β quantified from aspirates, as previously described^{44,45}.

Statistical analyses

We performed statistical analyses and graphical presentation using GraphPad Prism 8 (GraphPad Software, USA). Statistically significant differences between groups were determined using the Mann-Whitney U test, and the data is summarised as median with interquartile ranges (IQR). Categorical data were summarised as proportions and compared using the Fisher's exact test, with effect size reported as Relative Risk.

Results

Demographic characteristics of study population

A total of 47 HIV-uninfected children were recruited, comprising of 21 asymptomatic healthy children and 26 influenza-like illness outpatients (Table 1). The asymptomatic healthy controls were predominantly female (71.4%), while those with an influenza-like illness were predominantly male (75.0%). Furthermore, the ILI group was relatively younger (1 – 4yrs, 80.8 vs. 47.6%, $p=0.029$), and the age eligible children were more likely to have received the 13-valent pneumococcal conjugate vaccine (PCV13) (84.6 vs. 52.4%, $p=0.025$). PCV13 was rolled out in Malawi in 2011.

ILI patients exhibit increased propensity for harbouring a viral organism and/or multiple microbes in nasal aspirates

Using an FTD multiplex real-time PCR, we detected viruses, bacteria and fungi in the nasal aspirates among the ILIs and asymptomatic healthy controls (Figure 1A). ILI patients were more likely than healthy controls to have a virus in their nasal aspirate (Relative risk (RR), 1.4 [95% CI 1.069-1.953], $p=0.0222$). The prevalence of ILI-associated pathogens including influenza virus, human rhinovirus and enterovirus was 11.5%, 11.5% and 23% in ILI patients, and 4.7%, 4.7% and 14.3% in healthy controls, respectively. Furthermore, ILI patients were more likely to have greater than four microbes per individual compared to the asymptomatic healthy controls (RR, 1.9 [95% CI 1.427-2.395], $p<0.0001$) (Figure 1B). These findings suggest that children with ILI are not only more likely to harbour a viral organism but also multiple respiratory microbes within the nasal mucosa.

ILI patients harbour higher levels of pro-inflammatory IL-8 in the URT than asymptomatic healthy controls

Having established that an ILI is associated with increased likelihood of detecting a viral organism and/or multiple microbes, we investigated the cytokine microenvironment in the upper respiratory tract (URT) by measuring the levels of pro- and anti-inflammatory cytokines in nasal aspirates of ILI patients and healthy controls. The levels of pro-inflammatory cytokine IL-8 were higher in nasal aspirates from children presenting with ILI, compared to healthy controls (median [IQR], 265.7 [0 – 452.3] vs. 0 [0 – 127.3] pg/ml; $p = 0.0154$) (Figure 2A). Furthermore, children with ILI were more likely to have detectable IL-8 than asymptomatic healthy controls (RR, 1.9 [95% CI 1.46 – 2.72], $p < 0.0001$). In contrast, the levels of IFN- γ , IL-10 and active TGF- β in nasal aspirates were similar between children presenting with ILI and healthy controls (all $p > 0.05$; Figure 2B-D). Collectively, these findings suggest that children presenting with ILI likely have ongoing URT inflammation.

ILI patients exhibit higher likelihood of greater pneumococcal carriage densities than asymptomatic healthy controls

Following observations that ILI patients were likely to have an inflamed URT mucosa, we determined whether this could impact pneumococcal carriage dynamics. Combining the pneumococcal carriage detection data from culture and *lytA* PCR on NP swabs the prevalence of carriage was higher in ILI patients than asymptomatic healthy controls (84.6 vs. 57.1%, $p = 0.037$) (Figure 3A). There was a strong concordance between culture and *lytA* PCR (Sensitivity 0.9310, Specificity 0.7647) (Table 2). The difference in prevalence between the two groups is likely due to age differences⁴⁶, however the ages of carriage positives were similar in both groups (median, range; 2 (1-9) vs. 3 (1 -9), $p = 0.4476$). There was also no difference in median bacterial density between ILI patients and

asymptomatic healthy controls (3.24 [2.23 – 3.50] vs 3.89 [3.06 – 4.37]; $p=0.1115$) (Figure 3B). There was a strong correlation between pneumococcal density as measured by *lytA* PCR and culture ($r=0.6800$, $p<0.0001$) (Supplementary Figure 1). However, further analysis revealed that children with ILI were more likely to harbour bacterial densities of $\geq 10^4$ cfu/ml than asymptomatic healthy controls (RR, 4.2 [95% CI 2.396 – 7.919], $p<0.0001$) (Figure 3C). Taken together, these findings suggest that having an ILI is associated with increased propensity for high pneumococcal carriage density.

Journal Pre-proof

Discussion

This study describes the relationship between having an influenza-like illness (ILI) in children and pneumococcal carriage. Pneumococcus is the leading cause of pneumonia and invasive bacterial infections in all ages, with the greatest incidence being in children under the age of 5⁴⁷⁻⁴⁹. In this study, ILI patients had higher likelihood of greater pneumococcal carriage densities than asymptomatic healthy controls. This is consistent with studies that have shown that viral infection driven local inflammation in the nasal mucosa is associated with increased pneumococcal carriage load^{25-27,29,31}. In line with the role of IL-8 in maintaining an inflammatory microenvironment at the site of infection⁵⁰, having an ILI was associated with high levels of IL-8. Inflammation leads to increased adherence of pneumococci to the nasal mucosal surface^{33,51}, but also clearance of pneumococci from the URT⁵². Recent work from the experimental human challenge model has demonstrated that prior nasal infection with live attenuated influenza virus (LAIV) induces inflammation and impairs innate immune function, leading to increased pneumococcal carriage densities²⁹. It is therefore plausible that local inflammation in the URT during an ILI episode promotes a conducive environment for pneumococcal survival and replication.

On the other hand, the high pneumococcal carriage density could be one of the aetiological factors for the development of an influenza-like illness. ILI in this study was defined by clinical presentation, following the WHO guidelines⁴². It is well known that ILI may be caused by both viral and bacterial infections, of which *S. pneumoniae* is a potential aetiological agent⁴⁰. In our cohort, we observed a high propensity for respiratory viral organisms in the ILI patients compared to healthy controls. One of the common pathogens associated with ILI is influenza virus^{37,53}. The prevalence of influenza virus in the patients with ILI was 11.5%, and this is consistent with previous studies in Malawi that reported influenza prevalence between 8.3% to 13.7% among patients with severe acute respiratory

illness (SARI) and community cases of ILI^{54,55}. The ILI patients were more likely to have harbour more than four potentially pathogenic respiratory organisms per individual in their nasal aspirate compared to healthy controls. This highlights the complexity of identifying the underlying infective aetiology of ILI in children in high transmission and disease-burdened settings.

Nevertheless, the lack of data on the definitive aetiological agents in our ILI patients constitutes a study limitation. It is clear from our study and others^{40,56,57} that the majority of ILI cases likely not caused by influenza but by other viruses or bacteria. Defining the aetiology of ILI in children in high transmission and disease-burdened settings like Malawi should be a research priority, as it could help in development of potential interventions to curb transmission of potentially pathogenic respiratory organisms in the community. The other limitation of study is the imbalance in our two study groups in terms of age and gender, which potentially skewed our pneumococcal carriage prevalence data. However, pneumococcal carriage density was unlikely impacted by age in our study, since the median age of the carriage positive children was similar between the two groups. Furthermore, we found similar carriage rates among the PCV-13 age eligible children, but we were not able to serotype the pneumococcus in order to elucidate its impact on vaccine serotypes.

Unexpectedly, we found relatively high prevalence of other pathogens, including notably *Salmonella* and *pneumocystis jirovecii*. For the bacterial genus *Salmonella*, we were not able to identify the organisms to species level in order to differentiate between pathogenic and commensal organisms. In Malawi, at least 10.3% of bloodstream infections have been reported to be caused by *S. typhi*⁵⁸. Whilst, *Pneumocystis jirovecii* is estimated between 6.8 – 51%, to be the causative agent of children presenting with acute lower respiratory infection in Africa^{59–62}.

In conclusion, we have demonstrated that having an ILI is associated with increased propensity for high pneumococcal carriage density in children. These findings have potential implications in the development of interventions to curb pneumococcal disease and transmission, since high-density pneumococcal carriage is an important risk factor for pneumonia and transmission.

Journal Pre-proof

Author Contributions

AL, AK, KCJ and DRN conceived the study. TKN, AL, TDS, NF, RH, DE, AK, KCJ, DRN contributed reagents. TKN, AL, LSK, DtB, KCJ and DRN performed the experiments. TKN, AL, KCJ, DRN performed the data analysis. TKN and AL drafted the manuscript. TKN, AL, TDS, LSK, NF, RH, DE, AK, KCJ and DRN critically revised successive versions of the paper and approved the final version.

Acknowledgements

The authors thank all study participants, the clinical team (Mrs Mercy Juma, Mr Andrew Sigoloti, Ms Chifundo Kondoni) and supporting staff of Malawi-Liverpool-Wellcome Trust Clinical Research Programme and Queen Elizabeth Central Hospital for their support and cooperation during the study.

Conflict of interest

We declare no competing interests.

Funding

This work was supported by the CDC through a cooperative agreement (grant 5U01CK000146-04). TKN is supported by training grant awarded as part of the Wellcome Strategic award number 101113/Z/13/Z084 to The Malawi-Liverpool-Wellcome Trust Clinical Research Programme. KCJ was supported by the Wellcome (UK) through an Intermediate Fellowship number 105831/Z/14/Z. DRN is supported by a Sir Henry Dale Fellowship jointly funded by the Wellcome Trust and the Royal Society (grant number 204457/Z/16/Z). AL and AK were supported by a UK Medical Research Council Programme Grant (MR/P011284/1). RSH, NF and TS are supported by the National Institute for Health Research (NIHR) Global Health Research Unit on Mucosal Pathogens using UK aid from the UK Government. The views expressed in this publication are those of the author(s) and not necessarily those of the NIHR or the Department of Health and Social Care”.

References

1. Everett DB, Mukaka M, Denis B, Gordon SB, Carrol ED, van Oosterhout JJ, et al. Ten years of surveillance for invasive *Streptococcus pneumoniae* during the era of antiretroviral scale-up and cotrimoxazole prophylaxis in Malawi. *PLoS One* **2011**;6:e17765.
2. Wall EC, Cartwright K, Scarborough M, Ajdukiewicz KM, Goodson P, Mwambene J, et al. High mortality amongst adolescents and adults with bacterial meningitis in sub-Saharan Africa: An analysis of 715 cases from Malawi. *PLoS One* **2013**;8:e69783.
3. Wall E.C., B.Everett D., Mukaka M., Bar-Zeev N., Feasey N., Jahn A., et al. Bacterial meningitis in Malawian adults, adolescents, and children during the era of antiretroviral scale-up and *Haemophilus influenzae* type B vaccination, 2000-2012. *Clin Infect Dis* **2014**;58:e137–45.
4. Aston SJ, Ho A, Jary H, Huwa J, Mitchell T, Ibitoye S, et al. Etiology and risk factors for mortality in an adult community-acquired pneumonia cohort in Malawi. *Am J Respir Crit Care Med* **2019**;200:359-369.
5. Carrol ED, Mankhambo LA, Jeffers G, Parker D, Guiver M, Newland P, et al. The diagnostic and prognostic accuracy of five markers of serious bacterial infection in Malawian children with signs of severe infection. *PLoS One* **2009**;4:e6621.
6. Meiring S, Cohen C, Quan V, de Gouveia L, Feldman C, Karstaedt A, et al. HIV infection and the epidemiology of invasive pneumococcal disease (IPD) in South African adults and older children prior to the introduction of a pneumococcal conjugate vaccine (PCV). *PLoS One* **2016**;11:e0149104.
7. Kalata NL, Nyazika TK, Swarhout TD, Everett D, French N, Heyderman RS, et al. Pneumococcal pneumonia and carriage in Africa before and after introduction of pneumococcal conjugate vaccines, 2000–2019: protocol for systematic review. *BMJ Open* **2019**;9:e030981.
8. Richards L, Ferreira DM, Miyaji EN, Andrew PW, Kadioglu A. The immunising effect of pneumococcal nasopharyngeal colonisation; protection against future colonisation and fatal invasive disease. *Immunobiology* **2010**;215:251–63.
9. Iroh Tam P-Y, Thielen BK, Obaro SK, Brearley AM, Kaizer AM, Chu H, et al. Childhood pneumococcal disease in Africa – A systematic review and meta-analysis of incidence, serotype distribution, and antimicrobial susceptibility. *Vaccine* **2017**;35:1817–27.
10. Ngocho JS, Magoma B, Olomi GA, Mahande MJ, Msuya SE, de Jonge MI, et al. Effectiveness of pneumococcal conjugate vaccines against invasive pneumococcal disease among children under five years of age in Africa: A systematic review. *PLoS One* **2019**;14:e0212295.
11. Tan TQ. Pediatric invasive pneumococcal disease in the United States in the era of pneumococcal conjugate vaccines. *Clin Microbiol Rev* **2012**;25:409–19.
12. Carrol ED, Guiver M, Nkhoma S, Mankhambo LA, Marsh J, Balmer P, et al. High pneumococcal DNA loads are associated with mortality in Malawian children with invasive pneumococcal disease: *Pediatr Infect Dis J* **2007**;26:416–22.
13. Cornick JE, Everett DB, Broughton C, Denis BB, Banda DL, Carrol ED, et al. Invasive *Streptococcus pneumoniae* in children, Malawi, 2004–2006. *Emerg Infect Dis* **2011**;17:1107–9.

14. Wall EC, Everett DB, Mukaka M, Bar-Zeev N, Feasey N, Jahn A, et al. Bacterial meningitis in Malawian adults, adolescents, and children during the era of antiretroviral scale-up and *Haemophilus influenzae* type b vaccination, 2000-2012. *Clin Infect Dis Off Publ Infect Dis Soc Am* **2014**;58:e137-145.
15. Swarthout TD, Fronterre C, Lourenço J, Obolski U, Gori A, Bar-Zeev N, et al. High residual carriage of vaccine-serotype *Streptococcus pneumoniae* after introduction of pneumococcal conjugate vaccine in Malawi. *Nat. Commun* **2020**;11:2222.
16. Ding T, Song T, Zhou B, Geber A, Ma Y, Zhang L, et al. Microbial composition of the human nasopharynx varies according to influenza virus type and vaccination status. *mBio* **2019**;10:e01296-19.
17. Kumpitsch C, Koskinen K, Schöpf V, Moissl-Eichinger C. The microbiome of the upper respiratory tract in health and disease. *BMC Biol* **2019**;17:87.
18. Schenck LP, Surette MG, Bowdish DME. Composition and immunological significance of the upper respiratory tract microbiota. *FEBS Lett* **2016**;590:3705–20.
19. de Steenhuijsen Piters WAA, Sanders EAM, Bogaert D. The role of the local microbial ecosystem in respiratory health and disease. *Philos Trans R Soc B Biol Sci* **2015**;370:20140294.
20. Thevaranjan N, Whelan FJ, Puchta A, Ashu E, Rossi L, Surette MG, et al. *Streptococcus pneumoniae* colonization disrupts the microbial community within the upper respiratory tract of aging mice. *Infect Immun* **2016**;84(4):906–16.
21. Heinsbroek E, Tafatatha T, Phiri A, Swarthout TD, Alaerts M, Crampin AC, et al. Pneumococcal carriage in households in Karonga District, Malawi, before and after introduction of 13-valent pneumococcal conjugate vaccination. *Vaccine* **2018**;36(48):7369–76.
22. Hibbing ME, Fuqua C, Parsek MR, Peterson SB. Bacterial competition: surviving and thriving in the microbial jungle. *Nat Rev Microbiol* **2010**;8:15–25.
23. Shak JR, Vidal JE, Klugman KP. Influence of bacterial interactions on pneumococcal colonization of the nasopharynx. *Trends Microbiol* **2013**;21:129–35.
24. Grijalva CG, Griffin MR, Edwards KM, Williams JV, Gil AI, Verastegui H, et al. The Role of influenza and parainfluenza infections in nasopharyngeal pneumococcal acquisition among young children. *Clin Infect Dis* **2014**;58:1369–76.
25. Howard LM, Zhu Y, Griffin MR, Edwards KM, Williams JV, Gil AI, et al. Nasopharyngeal pneumococcal density during asymptomatic respiratory virus infection and risk for subsequent acute respiratory illness. *Emerg Infect Dis* **2019**;25:2040–7.
26. Morpeth SC, Munywoki P, Hammitt LL, Bett A, Bottomley C, Onyango CO, et al. Impact of viral upper respiratory tract infection on the concentration of nasopharyngeal pneumococcal carriage among Kenyan children. *Sci Rep* **2018**;8:1–8.
27. Vissers M, Ahout IM, van den Kieboom CH, van der Gaast de Jongh CE, Groh L, Cremers AJ, et al. High pneumococcal density correlates with more mucosal inflammation and reduced respiratory syncytial virus disease severity in infants. *BMC Infect Dis* **2016**;16:129.
28. Short KR, Reading PC, Wang N, Diavatopoulos DA, Wijburg OL. Increased nasopharyngeal bacterial titers and local inflammation facilitate transmission of *Streptococcus pneumoniae*. *mBio*. **2012**;3:e00255-12.

29. Jochems SP, Marcon F, Carniel BF, Holloway M, Mitsi E, Smith E, et al. Inflammation induced by influenza virus impairs human innate immune control of pneumococcus. *Nat Immunol* **2018**;19:1299–1308.
30. Murad C, Dunne EM, Sudigdoadi S, Fadlyana E, Tarigan R, Pell CL, et al. Pneumococcal carriage, density, and co-colonization dynamics: A longitudinal study in Indonesian infants. *Int J Infect Dis* **2019**;86:73–81.
31. Wolter N, Tempia S, Cohen C, Madhi SA, Venter M, Moyes J, et al. High Nasopharyngeal pneumococcal density, increased by viral coinfection, is associated with invasive pneumococcal pneumonia. *J Infect Dis* **2014**;210:1649–57.
32. Vu HTT, Yoshida LM, Suzuki M, Nguyen HAT, Nguyen CDL, Nguyen ATT, et al. Association between nasopharyngeal load of *Streptococcus pneumoniae*, viral coinfection, and radiologically confirmed pneumonia in Vietnamese children. *Pediatr Infect Dis J* **2011**;30:11–8.
33. Jochems SP, Weiser JN, Malley R, Ferreira DM. The immunological mechanisms that control pneumococcal carriage. *PLoS Pathog* **2017**;13:e1006665.
34. Moreno AT, Oliveira MLS, Ho PL, Vadesilho CFM, Palma GMP, Ferreira JMC, et al. Cross-reactivity of antiPneumococcal surface protein C (PspC) antibodies with different strains and evaluation of inhibition of human complement factor H and secretory IgA binding via PspC. *Clin Vaccine Immunol* **2012**;19:499–507.
35. Li Y, Jin L, Chen T. The Effects of secretory IgA in the mucosal immune system. *BioMed Res Int* **2020**;2020:1–6.
36. Maestro B, Sanz J. Choline binding proteins from *Streptococcus pneumoniae*: A dual role as enzybiotics and targets for the design of new antimicrobials. *Antibiotics* **2016**;5:21.
37. Rudge JW, Inthaphone N, Pavlicek R, Paboriboune P, Flaissier B, Monidarin C, et al. "Epidemiology and aetiology of influenza-like illness among households in metropolitan Vientiane, Lao PDR": A prospective, community-based cohort study. *PLoS One* **2019**;14:e0214207.
38. Muscatello D, Barr M, Thackway SV, MacIntyre CR. Epidemiology of influenza-like Illness during pandemic (H1N1) 2009, New South Wales, Australia. *Emerg Infect Dis* **2011**;17:1240–7.
39. Azziz-Baumgartner E, Alamgir A, Rahman M, Homaira N, Sohel BM, Sharker MY, et al. Incidence of influenza-like illness and severe acute respiratory infection during three influenza seasons in Bangladesh, 2008–2010. *Bull World Health Organ* **2012**;90:12–9.
40. Hui DS, Woo J, Hui E, Foo A, Ip M, To K-W, et al. Influenza-like illness in residential care homes: a study of the incidence, aetiological agents, natural history and health resource utilisation. *Thorax* **2008**;63:690–7.
41. Taylor S, Lopez P, Weckx L, Borja-Tabora C, Ulloa-Gutierrez R, Lazcano-Ponce E, et al. Respiratory viruses and influenza-like illness: Epidemiology and outcomes in children aged 6 months to 10 years in a multi-country population sample. *J Infect* **2017**;74:29–41.
42. Fitzner J, Qasmieh S, Mounts AW, Alexander B, Besselaar T, Briand S, et al. Revision of clinical case definitions: influenza-like illness and severe acute respiratory infection. *Bull World Health Organ* **2018**;96:122–8.

43. Gritzfeld JF, Cremers AJH, Ferwerda G, Ferreira DM, Kadioglu A, Hermans PWM, et al. Density and duration of experimental human pneumococcal carriage. *Clin Microbiol Infect* **2014**;20:O1145–51.
44. Abe M, Harpel JG, Metz CN, Nunes I, Loskutoff DJ, Rifkin DB. An assay for Transforming Growth Factor-Beta using cells transfected with a plasminogen activator inhibitor-1 promoter-luciferase construct. *Anal Biochem* **1994**;216:276–84.
45. Neill DR, Coward WR, Gritzfeld JF, Richards L, Garcia-Garcia FJ, Dotor J, et al. Density and duration of pneumococcal carriage is maintained by Transforming Growth Factor β 1 and T regulatory cells. *Am J Respir Crit Care Med* **2014**;189:1250–9.
46. Roca A, Bottomley C, Hill PC, Bojang A, Egere U, Antonio M, et al. Effect of age and vaccination with a pneumococcal conjugate vaccine on the density of pneumococcal nasopharyngeal carriage. *Clin Infect Dis* **2012**;55:816–24.
47. Azzari C, Cortimiglia M, Nieddu F, Moriondo M, Indolfi G, Mattei R, et al. Pneumococcal serotype distribution in adults with invasive disease and in carrier children in Italy: Should we expect herd protection of adults through infants' vaccination? *Hum Vaccines Immunother* **2016**;12:344–50.
48. Troeger C, Forouzanfar M, Rao PC, Khalil I, Brown A, Swartz S, et al. Estimates of the global, regional, and national morbidity, mortality, and aetiologies of lower respiratory tract infections in 195 countries: a systematic analysis for the Global Burden of Disease Study 2015. *Lancet Infect Dis* **2017**;17:1133–61.
49. Wahl B, O'Brien KL, Greenbaum A, Majumder A, Liu L, Chu Y, et al. Burden of *Streptococcus pneumoniae* and *Haemophilus influenzae* type b disease in children in the era of conjugate vaccines: global, regional, and national estimates for 2000–15. *Lancet Glob Health* **2018**;6:e744–57.
50. Weight CM, Venturini C, Sherin P, Jochems SP, Reiné J, Nikolaou E, et al. Microinvasion by *Streptococcus pneumoniae* induces epithelial innate immunity during colonisation at the human mucosal surface. *Nat Commun* 2019;10:1–15.
51. Adamou JE, Wizemann TM, Barren P, Langermann S. Adherence of *Streptococcus pneumoniae* to Human Bronchial Epithelial Cells (BEAS-2B). *Infect Immun* **1998**;66:820–2.
52. van Rossum AMC, Lysenko ES, Weiser JN. Host and bacterial factors contributing to the clearance of colonization by *Streptococcus pneumoniae* in a murine model. *Infect Immun* **2005**;73:7718–26.
53. Renois F, Talmud D, Huguenin A, Moutte L, Strady C, Cousson J, et al. Rapid detection of respiratory tract viral infections and coinfections in patients with influenza-like illnesses by use of reverse transcription-PCR DNA microarray systems. *J Clin Microbiol* **2010**;48(11):3836–42.
54. Peterson I, Bar-Zeev N, Kennedy N, Ho A, Newberry L, SanJoaquin MA, et al. Respiratory virus-associated severe acute respiratory illness and viral clustering in Malawian children in a setting with a high prevalence of HIV infection, malaria, and malnutrition. *J Infect Dis* **2016**;214:1700–11.
55. Ho A, Aston SJ, Jary H, Mitchell T, Alaerts M, Menyere M, et al. Impact of human immunodeficiency virus on the burden and severity of influenza illness in Malawian adults: a prospective cohort and parallel case-control study. *Clin Infect Dis* **2018**;66:865–76.

56. Assane D, Makhtar C, Abdoulaye D, Amary F, Djibril B, Amadou D, et al. Viral and bacterial etiologies of acute respiratory infections among children under 5 years in Senegal. *Microbiol Insights* **2018**;11:1–5.
57. Setiawaty V, Puspa KD, Adam K, Pangesti KN. Upper respiratory tract bacteria in Influenza-like illness cases in Indonesia using multiplex PCR method. *HSJI* **2013**;4:83–6.
58. Musicha P, Cornick JE, Bar-Zeev N, French N, Masesa C, Denis B, et al. Trends in antimicrobial resistance in bloodstream infection isolates at a large urban hospital in Malawi (1998–2016): a surveillance study. *Lancet Infect Dis* **2017**;17:1042–52.
59. Lanaspá M, O’Callaghan-Gordo C, Machevo S, Madrid L, Nhampossa T, Acácio S, et al. High prevalence of *Pneumocystis jirovecii* pneumonia among Mozambican children <5 years of age admitted to hospital with clinical severe pneumonia. *Clin Microbiol Infect* **2015**;21:1018.e9-1018.e15.
60. Graham SM, Mtitimila EI, Kamanga HS, Walsh AL, Hart CA, Molyneux ME. Clinical presentation and outcome of *Pneumocystis carinii* pneumonia in Malawian children. *The Lancet* **2000**;355:369–73.
61. Morrow BM, Samuel CM, Zampoli M, Whitelaw A, Zar HJ. Pneumocystis pneumonia in South African children diagnosed by molecular methods. *BMC Res Notes* **2014**;7:26.
62. Morris A, Lundgren JD, Masur H, Walzer PD, Hanson DL, Frederick T, et al. Current epidemiology of Pneumocystis pneumonia. *Emerg Infect Dis* **2004**;10:1713–20.

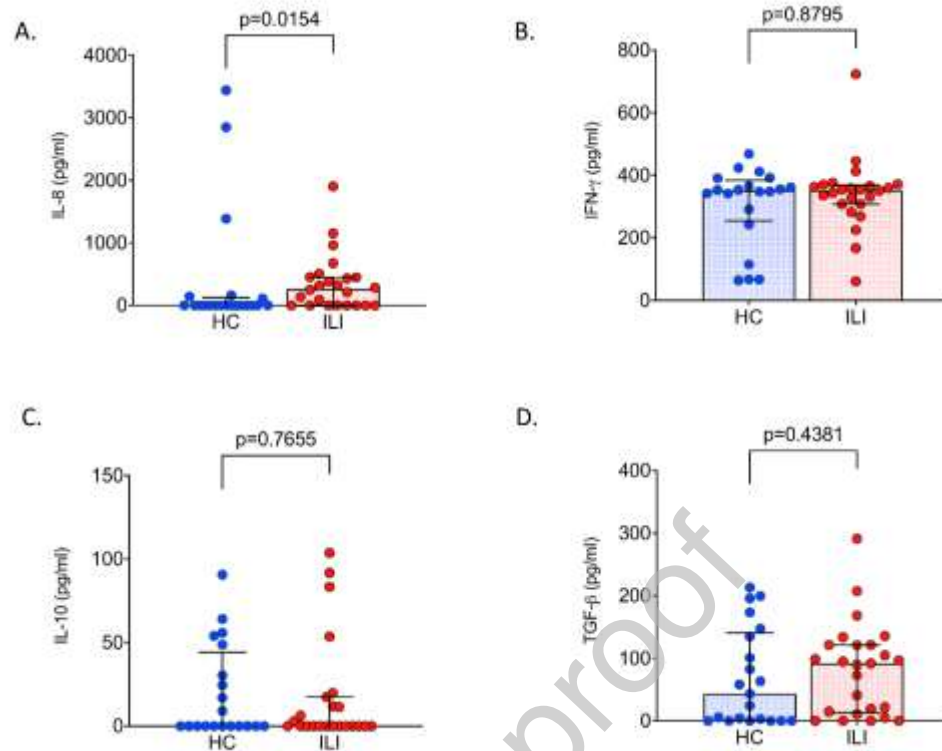


Figure 2: Cytokine levels in nasal aspirates of ILI patients and healthy controls. Levels of IL-8, FN- γ and IL-10 were measured by ELISA in nasal aspirates from ILI patients and healthy controls. Active TGF- β was also measured using a luciferase-reporting transformed mink lung epithelial cell assay. A) Concentration of IL-8 in nasal aspirates in healthy children and those with an ILI. B) Concentration of IFN- γ in nasal aspirates in healthy children and those with an ILI. C) Concentration of IL-10 in nasal aspirates in healthy children and those with an ILI. D) Concentration of active TGF- β in nasal aspirates in healthy children and those with an ILI. Data were analysed using Mann Whitney test. The bars represent median. ILI was defined as Influenza-like-illness according to WHO syndromic case-definitions. ILI= Influenza-like illness (cases) (n=26); HC= Healthy child (control) (n=21).

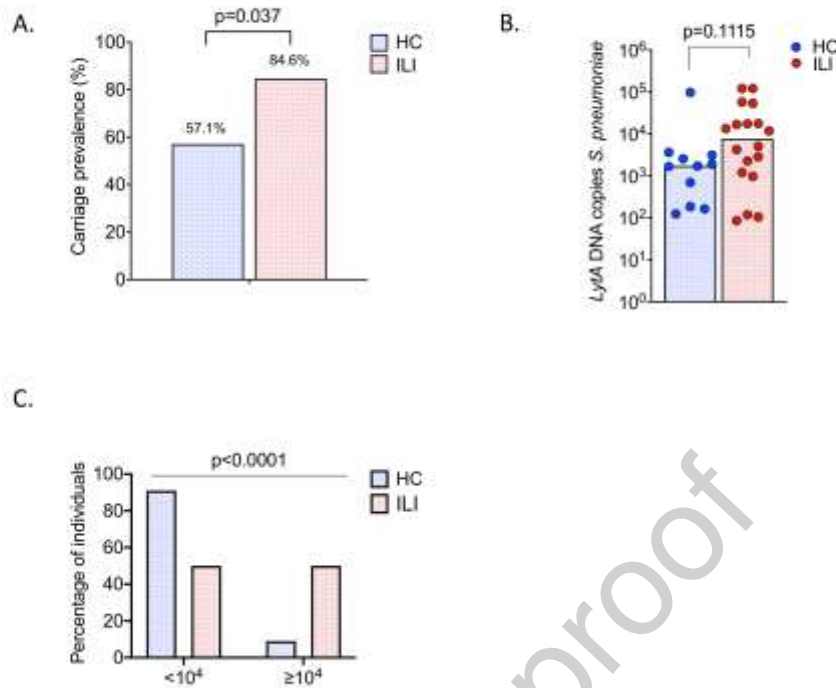


Figure 3: Point prevalence of *Streptococcus pneumoniae* nasopharyngeal carriage and density in ILI patients and healthy controls. Quantitative PCR targeting *lytA* gene and culture were used to determine pneumococcal carriage rates and carriage densities in nasal swabs. FTD multiplex PCR was used to detect 33 respiratory organisms in nasal aspirates. A) *S. pneumoniae* carriage prevalence among healthy controls and ILI children based on aggregation of culture and *lytA* PCR. Data were analysed using a Chi-square test. HC (n=21), ILI (n=26). B) *S. pneumoniae* carriage densities between ILI patients and healthy controls. Data were analysed using Mann Whitney test. The bars represent median. HC (n=11), ILI (n=16) C). *S. pneumoniae* carriage densities [(medium/low carriage (<10⁴ copies) vs high carriage ≥10⁴)] between ILI patients and healthy controls. Data was analysed using a Fischer's exact test. HC (n=11), ILI (n=16). ILI was defined as Influenza-like-illness

according to WHO syndromic case-definitions. ILI= Influenza-like illness (cases); HC= Healthy child (control)

Journal Pre-proof

TABLES

Table 1. Demographic characteristics of study population in relation to ILI status.

Variable	All participants ^a (n=47)	Healthy controls (n = 21)	ILI ^b (n = 26)	p value ^c
Age, years, median (IQR)	3 (2 – 6)	5 (2 – 6)	2 (1 – 4)	0.078
1–<5	31	10 (47.6)	21 (80.8)	0.029
≥5–10	16	11 (52.4)	5 (19.2)	
Sex				0.245
Female	29 (61.7)	15 (71.4)	14 (53.8)	
PCV-vaccinated ^d				0.025
Yes	33 (70.2)	11 (52.4)	22 (84.6)	

^aAll participants were HIV negative.

^bILI was defined as Influenza-like-illness according to WHO syndromic case-definitions.

^cFishers Exact test of categorical data; t-test for continuous data.

^dPCV pneumococcal conjugate vaccine – all 3 routine scheduled doses

Table 2: Relationship between culture and *LytA* PCR

	Culture Positive	Culture Negative	Total	Diagnostic accuracy
<i>LytA</i> Positive	27 (93.1%)	4 (23.5%)	31 (67.4%)	<u>Sensitivity</u> 0.9310 (95% CI 0.7804 – 0.9877) <u>Positive predictive value</u> 0.8710 (95% CI 0.7115 – 0.9487)
<i>LytA</i> Negative	2 (6.9%)	13 (76.5%)	15 (22.6%)	<u>Specificity</u> 0.7647 (95% CI 0.5274 – 0.9044) <u>Negative predictive value</u> 0.8667 (95% CI 0.6212 – 0.9763)
Total	29 (63.0%)	17 (37.0%)	46 (100%)	