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

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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.

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 pneumonia.[1–5] 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 and 5yrs.[6–8] 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 time,[15] and is influenced by the microbial composition of the upper respiratory tract (URT), including viral co-infections.[16–21] 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,

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influenza virus, respiratory syncytial virus (RSV) and parainfluenza virus have been reported.[24–26]

Viral-induced local inflammation in the nasal mucosa has been implicated as an important factor that promotes pneumococcal carriage and transmission.[27–29] 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 infection.[27] 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 surface.[33–36] 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 children,[37–39] with respiratory viral and bacterial pathogens reported as the key aetiological 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 °C), cough but not requiring hospitalisation.[42] 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 (FLOQSwabs™, 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 s 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 (100–120 mmHg for children; 120–150 mmHg 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-h of collection.

Streptococcus pneumoniae *culture and* lytA *PCR*

Nasopharyngeal (NP) swabs, after collection and prior to initial freezing, were vortexed and 100 µl 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% CO_2. Plates showing no *S. pneumoniae* growth were incubated for a further 24-h 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 $<$ 14 mm. 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 previously.[43] 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 300 µl aliquots of the nasal aspirate by manual extraction using the QIAamp DNA Mini Kit (Qiagen, Manchester, UK), according to manufacturer's instructions. 10 µl 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; Chlamydophila 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 $\leq$ 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

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)	

[a] All participants were HIV negative.
[b] ILI was defined as Influenza-like-illness according to WHO syndromic case-definitions.
[c] Fishers Exact test of categorical data; *t*-test for continuous data.
[d] PCV pneumococcal conjugate vaccine – all 3 routine scheduled doses.

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 (Fig. 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], $p < 0.0001$) (Fig. 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; $p = 0.0154$) (Fig. 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 $p > 0.05$; Fig. 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%, $p = 0.037$) (Fig. 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 differences, [46] 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]; $p = 0.1115$) (Fig. 3B). There was a strong correlation between pneumococcal density as measured by *lytA* PCR and culture ($r = 0.6800$, $p < 0.0001$) (Supplementary Fig. 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], $p < 0.0001$) (Fig. 3C). Taken together, these findings suggest that having an ILI is associated with increased propensity for high pneumococcal carriage density.

Discussion

This study describes the relationship between having an influenza-like illness (ILI) in children and pneumococcal carriage.

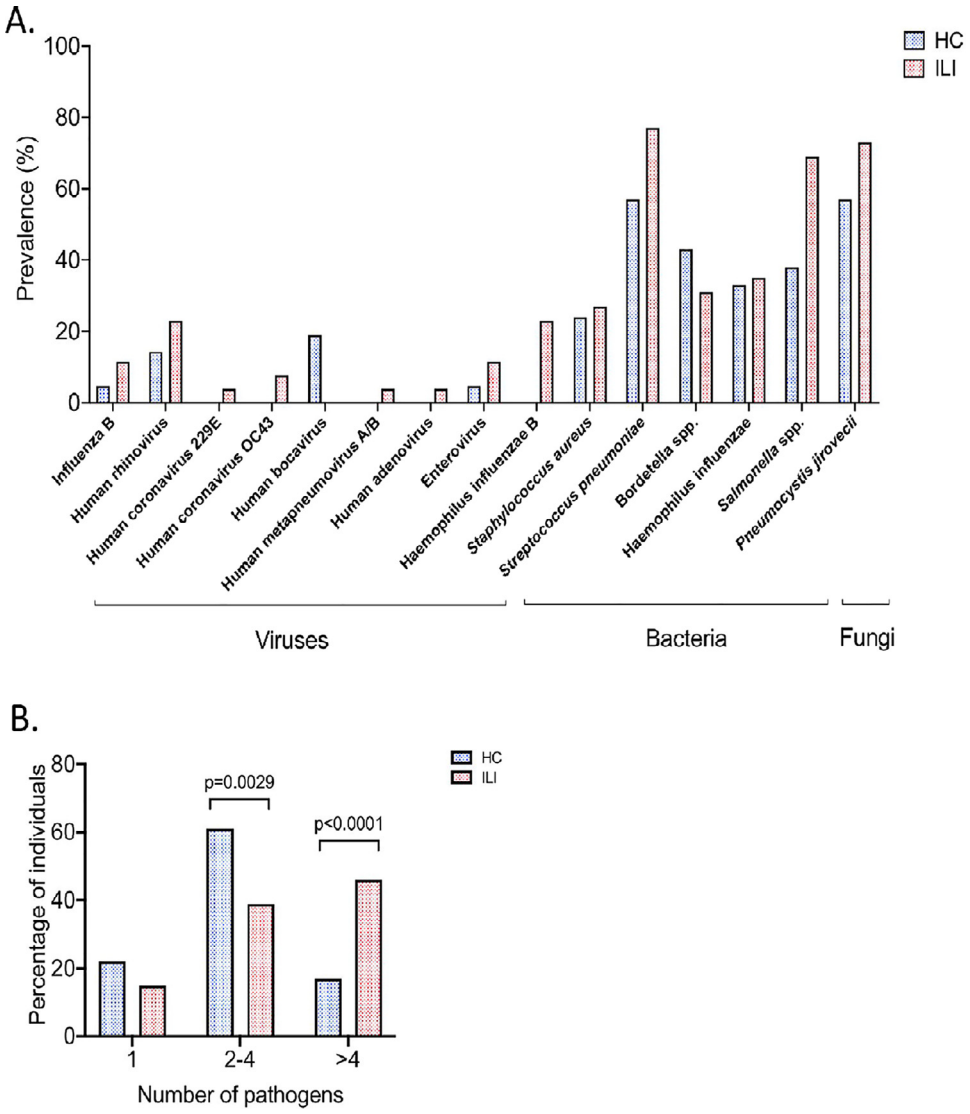

Fig. 1. *Prevalence of respiratory pathogens in nasal aspirates of ILI patients and healthy controls.* A multiplex real-time PCR was used to detect 33 respiratory pathogens in nasal aspirates of children with an influenza-like illness and healthy controls. A) Prevalence of detectable viral, bacterial and fungal pathogens in nasal aspirates. B) Prevalence of multiple pathogens in nasal aspirates of children with an influenza-like illness compared to healthy controls. Chi-square tests was used to compare the two groups. ILI= Influenza-like illness (cases) ($n=26$); HC= Healthy child (control) ($n=21$).

Table 2
Relationship between culture and *LytA* PCR.

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

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 5.[47–49] 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 infection, [50] 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 URT. [52] 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 densities.[29] It

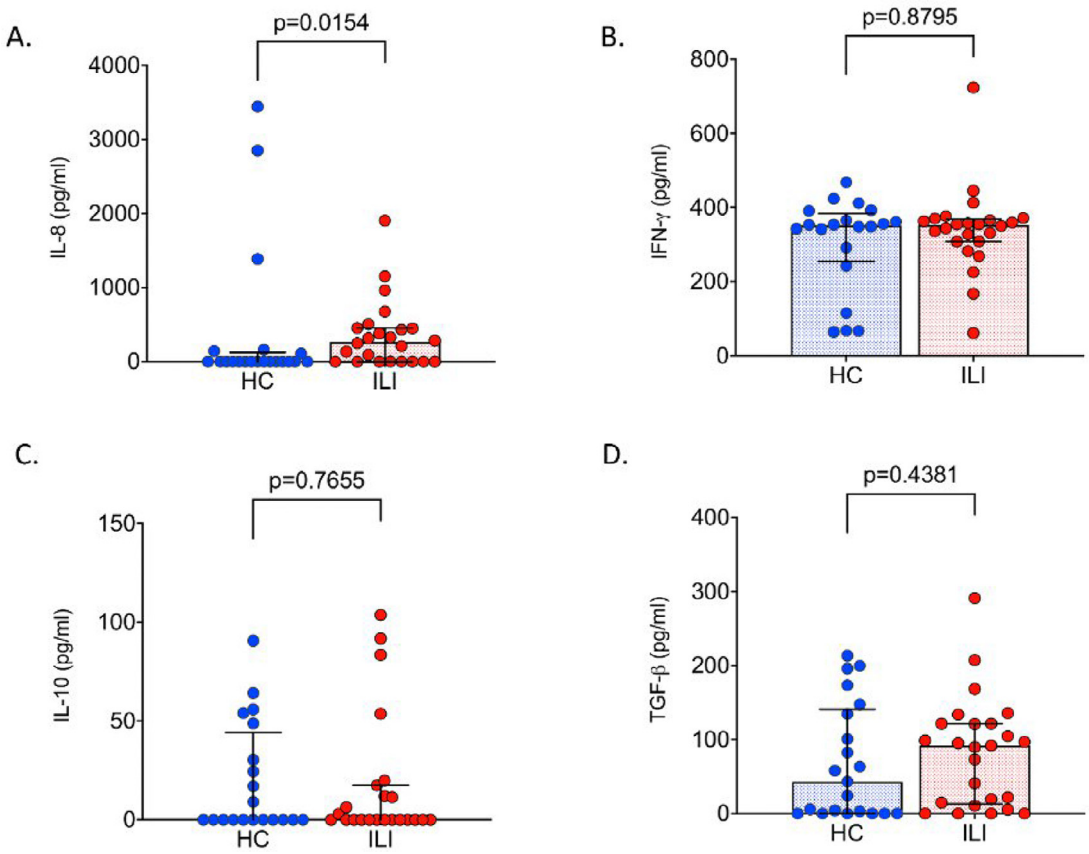

Fig. 2. *Cytokine levels in nasal aspirates of ILI patients and healthy controls.* Levels of IL-8, FN-γ and IL-10 were measured by ELISA in nasal aspirates from ILI patients and healthy controls. Active TGF-β was also measured using a luciferase-reporting transformed mink lung epithelial cell assay. 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) ($n=21$).

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 guidelines.[42] It is well known that ILI may be caused by both viral and bacterial infections, of which *S. pneumoniae* is a potential aetiological agent.[40] 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% and 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.

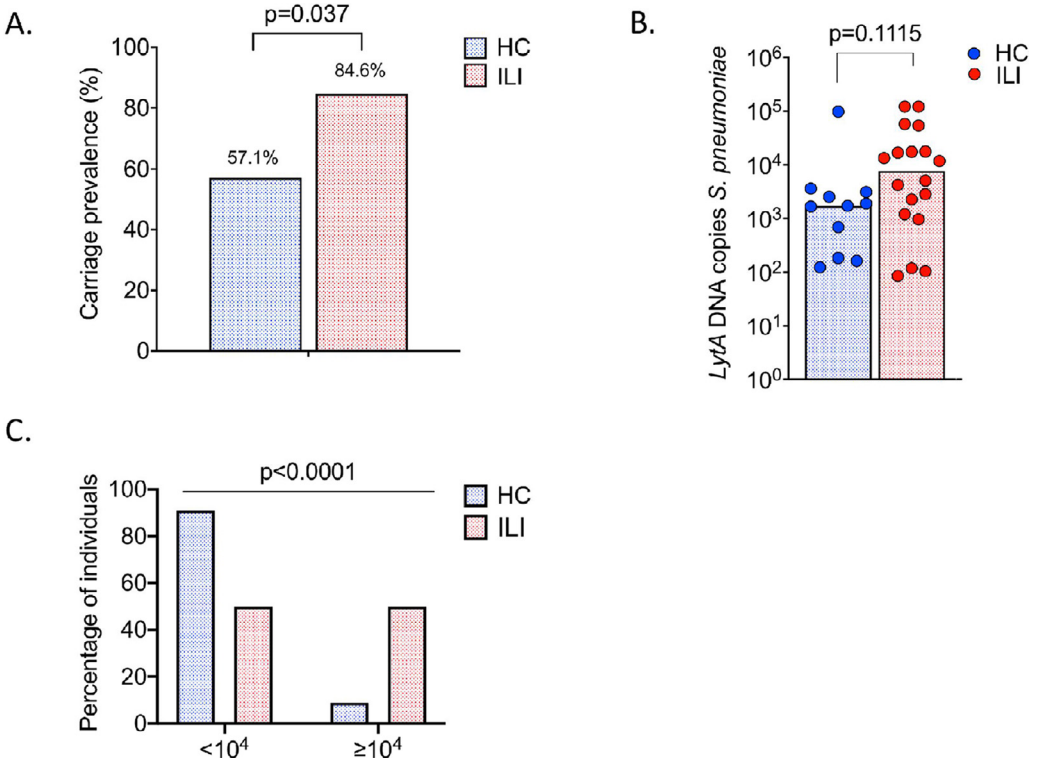

Fig. 3. *Point prevalence of Streptococcus pneumoniae nasopharyngeal carriage and density in ILI patients and healthy controls.* Quantitative PCR targeting *lytA* gene and culture were used to determine pneumococcal carriage rates and carriage densities in nasal swabs. FTD multiplex PCR was used to detect 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 ($<10^4$ copies) vs. high carriage $\geq 10^4$)] 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).

Declaration of Competing Interest

We declare no competing interests.

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.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jinf.2020.06.079.

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