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Population Dynamics of Nasopharyngeal Bacterial Pathogens in Febrile Tanzanian Children

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ABSTRACT

Population Dynamics of Nasopharyngeal Bacterial Pathogens in Febrile Tanzanian Children

By David Alfa

Background: The nasopharynx of children serves as a reservoir of bacterial pathogens such as *Streptococcus pneumoniae (Sp)*, *Haemophilus influenzae (Hi), Staphylococcus aureus (Sa), Moraxella catarrhalis (Mc)* and *Neisseria meningitidis (Nm)*. These pathogens are responsible for most of the global burden of bacterial respiratory diseases, otitis media and meningitis in children. Previous studies in healthy children showed these pathogens interact with each other within the nasopharynx in a competitive or synergistic manner. However, little to nothing is known about population dynamics of these bacteria in the nasopharynx of febrile children and its biological importance for the disease process. Therefore, our studies analyzed for the first time the bacterial density, using molecular approaches, of all these pathogens in the nasopharynx of febrile African children.

Methods: This was a cross sectional study where nasopharyngeal (NP) swabs were collected from febrile Tanzanian children (N= 999) aged 2 months to 10 years old. Identification and quantification of bacterial densities was done by quantitative (q)PCR analysis utilizing template DNA purified from those NP swabs. Logistic regression models were employed (after dichotomizing bacterial densities into absence or presence of targeted gene) to determine patterns of associations between bacterial species and potential association between patterns of densities with disease processes or risk factors associated to febrile illness. Spearman correlation coefficients were used to assess correlations among bacterial densities.

Results: The overall prevalence of these bacterial species in all febrile children was: *Sp* (80.8%), *Hi* (74.2%), *Sa* (23.3%), *Mc* (90.9%) and *Nm* (50.1%). Most children colonized by *Sp, Hi* and *Mc* had high densities of these bacteria in their nasopharynx. Concurrent colonization in this population by more than one bacterial specie was distributed as follows: 2 species (12.5%), 3 species (32.5 %), 4 species (33.8 %) and all five species (13.5 %) respectively. Near 65% of febrile children carried both *Sp* and *Mc* in the NP. Logistic regression models showed positive associations between colonization by *Sp*, *Hi* and *Mc*; *Sp* and *Hi* (OR 2.21, 95% CI 1.47-3.30, p-value <0.0001), *Sp* and *Mc* (OR 5.63, 95% CI 3.27- 9.70, p-value <0.0001) and between *Hi* and *Mc* (OR 3.76 95% CI 2.17-6.51, p-value <0.0001) while adjusting for age, sex, site of enrollment and acute respiratory infection status. A negative association was seen between colonization by *Sp* with *Sa* (adjusted OR 0.56, 95% CI 0.36-0.85, p-value <0.0001). Regression analysis also showed that age of the child was associated with colonization by *Nm* and site of enrollment was associated with colonization by *Sp, Hi* and *Mc*.

Conclusions: Studies within this thesis revealed high prevalence rates of *Sp*, *Hi* and *Mc* in febrile children. Most children (~92.4 %) will carry at least 2 bacterial pathogens with near 14% carrying all five pathogens. NP carriage of *Sp, Hi* and *Mc* was common in children with acute respiratory infections, whereas the prevalence of all these pathogens in the NP of children with other febrile illnesses, including pneumonia, was relatively low. Children enrolled in rural areas

were more densely colonized by *Sp, Hi* and *Mc* than those enrolled in the urban site. The strongest association was *Sp* and *Mc*.

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INTRODUCTION

Studies have shown that the nasopharynx of children can serve as an ecologic reservoir of respiratory bacterial pathogens such as *Streptococcus pneumoniae, Moraxella catarrhalis, Staphylococcus aureus, Haemophilus influenzae* and *Neisseria meningitidis*. These pathogens, following colonization of the nasopharynx, are the source of many childhood diseases such as ear infections, sinusitis, epiglottitis, pneumonia, endocarditis and bacteremia. Viruses can also join the microbial milieu as a prelude to secondary bacterial infections of the respiratory system.

However, as much as the knowledge of disease pathogenesis by these microorganisms is known, little is still known about the population dynamics, densities and interactions of these pathogens in the human nasopharynx of a febrile child and its effect on disease outcome in the respiratory tract or other anatomic sites. Therefore more knowledge about these pathogens in this group is required.

The complex milieu of these nasopharyngeal pathogens can be modified by factors such as the use of antimicrobial drugs, vaccines or surgical interventions such as tonsillectomies or adenoidectomies. These modifications may lead to changes in the interactions among microorganisms and/or cause synergisms of specific pathogens. Synergistic or competitive outcomes among these microorganisms ultimately affect the incidence of disease in children. Other factors that can modify the nasopharyngeal colonization dynamics in children include age, gender, presence of a febrile illness, history of breastfeeding, exposure to tobacco smoke or household indoor smoke, socioeconomic status (overcrowding) and immune/nutrition status of the child [1,2].

The nasopharynx and upper respiratory tract are commonly colonized by a wide range of organisms such as viruses, fungi, bacteria and parasites; however only a select group will cause infections in the respiratory tract. The mechanism by which an inhabitant of the nasopharynx becomes a pathogen is still under investigation and the motivation for this thesis. Globally, children acquire each year between two and five upper respiratory tract infections; most child hospitalizations and deaths are due to acute respiratory infections caused by pathogens investigated in this thesis.

Purpose statement

This study seeks firstly, to determine the colonization patterns and population densities of *S. pneumoniae, M. catarrhalis, S. aureus, H. influenzae and N. meningitidis* in the nasopharynx of Tanzanian children with febrile diseases. Secondly, it seeks to identify potential association(s) between populations of a specific pathogen in the nasopharynx of febrile children. Finally, to identify synergism of specific pathogens in the nasopharynx of children with febrile cases.

Study Hypothesis

We hypothesized that patterns of bacterial densities among respiratory pathogens colonizing the nasopharynx would predict different febrile illnesses and/or will be predictors of risk factors associated to those diseases.

Significance statement

In Africa, most febrile illnesses in children are generically treated with anti malarial medicines and these results in a high level of anti malarial consumption [3]. However, there is now an increasing awareness that not all febrile episodes are due to malaria and that other infectious diseases brought on by respiratory pathogens must be identified and tackled appropriately. At a time of rapidly decreasing malaria incidence rates, it is crucial to better understand the causes of non-malarial fevers, to improve case management of sick children. Determining the possible etiologies of fevers in African children will involve a comprehensive understanding of the population dynamics of respiratory pathogens which typically colonize the nasopharynx of these children. Such understanding would also help in designing therapeutic agents and vaccines which would target specific pathogenic agents without increasing the population of other unwanted pathogens which might cause further disease.

Developing countries usually lack efficient primary health care thereby high numbers of child mortality. These countries would greatly benefit from strategies for reducing diseases caused by pathogens colonizing the nasopharynx. Some of these strategies include: expanded immunization programs, developing more sensitive and inexpensive diagnostic tests and public health awareness campaigns are already being implemented and are helping to reduce burden of disease in these children.

LITERATURE REVIEW

The nasopharynx is the uppermost part of the throat behind the nose. It is a part of the pharynx which comprises three sections: the nasopharynx, oropharynx and the hypopharynx and is situated behind the nasal fossa inside the occipital bone. The nasopharynx is home to many bacterial microorganisms that begin colonizing it soon after birth which makes it an important ecological reservoir. Some of these micro-organisms can become pathogenic and following invasion of the host can cause disease.

The human nasopharynx is considered the niche from which important respiratory tract infections, such as pneumonia, originate. Several residents of the nasopharyngeal microbiome, including *S. pneumoniae, H. influenzae, M. catarrhalis, S. aureus* and *N. meningitidis*, are major contributors to disease in childhood. However, they are also common, transient colonizers of the nasopharynx of healthy children, especially in the youngest, whose immune systems are still maturing. Colonization of this niche in the upper respiratory tract appears to be a dynamic process of acquisition and elimination of various microbes, during which they interact with the host, its maturing immune system and each other. In a balanced state, this bacterial ecosystem is assumed to be beneficial for health, for example by stimulating the immune system and functioning as a protective barrier against invading pathogens [4].

Not much is known about the detailed structure of the nasopharyngeal bacterial flora especially in neonates and during early life as this flora is formed and subsequently becomes established during the first year of life. It is understood that the details of this bacterial flora differ between different individuals; however bacterial colonization trends are generally the same. A thorough understanding of how this bacterial flora develops, what contributions come

from parents or caregivers and regional influences is an important part of genomic research. This knowledge will undoubtedly improve the ability to prevent diseases caused by these pathogens in children.

Respiratory bacterial infections such as otitis media, epiglotitis, pneumonia, endocarditis and bacteremia in children are mostly caused by these bacterial colonizers of which the most common ones are the four previously listed. It is thought that probably all humans are colonized by these potential bacterial pathogens at least once during early life. In most cases, nasopharyngeal carriage of these potential pathogens is asymptomatic and identifiable disease only occurs in a percentage of persons who are colonized. Carriage rates for these bacteria vary widely in different populations. Some studies have shown that the prevalence of *S. pneumoniae* and *H. influenzae* in the nasopharnyx of children ranges from 13 – 85% and 6 – 80% respectively [6]. A study in New York, demonstrated that the carriage rate of *M. catarrhalis* was 66% among infants [26] while yet another study showed the prevalence of *S. aureus* in children to range between 10 – 35% [47]. Among healthy children in Cankaya municipality of Ankara province, a study determined the nasopharyngeal carriage rate for *N. meningitidis* was 10.4% [35].

For most respiratory bacterial infections to occur, colonization of the nasopharynx is an essential step. Viruses can also join the microbial milieu as a prelude to secondary bacterial infections of the respiratory tract.

Factors that influence the nasopharyngeal bacterial flora

Several factors are believed to affect the colonization of the nasopharynx by bacteria. Host factors, age, social factors, history of breastfeeding, seasonality and environmental factors are all thought to influence the dynamics of the nasopharyngeal bacterial milieu.

Local host immune status, upper/lower respiratory infections and otitis media are important in regulating the dynamics of pathogens through the interplay of acquisition and clearance of these pathogens. Organization of a host's defense against these pathogens would determine if the host is infected, type of strain of pathogen infected with and duration of infection. The integrity of the respiratory mucosa, anatomical defects such as the obstruction of the Eustachian tube, nutritional deficiencies and mucosal immune responses play an important role in affecting the colonization of the nasopharynx [2]. Otitis-prone children generate a poor local immune response to non-typeable *H. influenzae*, which leads to persistent and recurrent colonization. In contrast, children who become colonized briefly tend to generate a good local immune response and also serotypes of *S. pneumoniae* that are poorly immunogenic tend to colonize for longer periods [8].

Neto et al. also demonstrated that among the risk factors identified for carriage of respiratory pathogens was the presence of a lower respiratory infection. In children, the carriage of acute otitis media causing pathogens such as *S. pneumoniae*, non-typeable *H. influenzae* and *M. catarrhalis* increased significantly during episodes of otitis media compared with healthy periods. At the same time, non pathogens of the resident flora e.g. *S. viridians* declined in carriage: 65% vs. 22% [4]. This suggests that respiratory pathogens become relatively more important in the microenvironment of the nasopharynx during episodes of otitis media [4].

Studies have shown that age affects the colonization patterns of potential pathogenic bacteria in the nasopharynx. There is an increased risk of colonization by *S. pneumoniae* in children less than three years of age. It is understood that the duration of carriage of pneumococcal serotypes in children is inversely correlated with age. Poorly immunogenic serotypes can be carried in the nasopharynx of younger children for much longer than the more immunogenic types [5]. However, it is believed that young infants less than six months old are largely protected from pneumococcal carriage [5].

Social factors such as siblings, day care attendance and socioeconomic status also influence the risk of bacterial colonization of the nasopharynx [9]. Places such as daycares tend to be crowded areas and therefore facilitate horizontal transmission of bacterial respiratory pathogens among children and the community [10]. Low socioeconomic status in most cases is synonymous with poor hygiene, overcrowding and poor access to health care all of which are strong predictors of nasopharyngeal carriage and transmission of respiratory infections. A study in Australian Aboriginal infants attributed early bacterial colonization to high rates of cross infection due to overcrowding and poor hygiene [11].

Also, the type of bacterial pathogens colonizing the nasopharynx of infants and children differ by geography and socioeconomic status. Aboriginal infants are colonized by *H. influenzae* and *S. pneumoniae* as early as 10 and 20 days of age respectively while Finnish children were colonized by *S. pneumoniae* at 2 years of age. These results show that the carriage status in newborns and infants are greatly influenced by the socioeconomic status of the study population [6].

There are conflicting views about the impact of breastfeeding on nasopharyngeal bacterial colonization. One study conducted in India showed that two month old infants who

were fed colostrum were more likely to be colonized than those who did not [12]. Another study among children with acute otitis media showed that the prevalence of nontypeable *H. influenzae* (NTHi) in the nasopharynx was lower in breast fed infants than non breast fed infants. This might be because breastfeeding shows an association with higher levels of antibodies to NTHi antigens [13].

Some studies do not find any significant difference in the colonization rates between male and female infants although they did however report that Beta hemolytic streptococci were most likely to be isolated from male infants and that males tended to have a higher density of *S. aureus* than females [14]. The bacterial nasopharyngeal carriage appears to vary between seasons though the seasonal effects are not significant. The rate of colonization of the nasopharynx by *S. aureus* is more pronounced in the autumn and winter months in infants [14]. Also, the rate of nasopharyngeal carriage for *S. pneumoniae* increases during the winter season [15]. These seasonal trends might be due to weakened hosts' immunity due to preceding/coexisting viral infections acquired during the winter.

Streptococcus pneumoniae

Streptococcus pneumoniae is a Gram positive diplococcus which is often a normal commensal of the nasopharynx in children and adults. It is a major cause of morbidity and mortality in children less than 5 years of age especially in developing countries. It is implicated in both invasive (meningitis and septicemia) and non-invasive (pneumonia and otitis media). A study estimates that in 2011 there were 1.3 million deaths in children as a result of pneumonia.

This study also demonstrated that 18.3% of vaccine preventable severe pneumonia in children was due to *S. pneumoniae* [16].

There are over 90 known capsular serotypes described and whereas all serotypes may cause severe disease, a relatively limited number of serotypes cause the majority of invasive pneumococcal diseases. The serotypes currently included in existing pneumococcal conjugate vaccine (PCV) formulations account for 49-88% of deaths in children under 5 in Africa and Asia, where the morbidity and mortality of pneumococcal diseases are the highest, and where until recently, most children did not have access to current pneumococcal conjugate vaccines. Seven serotypes (1, 5, 6A, 14, 19F, and 23F) are the most common globally and account for the majority of invasive pneumococcal disease in every region [17]. The pneumococcal nasopharyngeal carriage rates among children in developing countries are generally two-to-three fold times higher than those found in children from developed countries and these carriage rates may involve multiple serotypes (or co-colonization) [5]. Co-colonization is an important event for pneumococcal evolution as it represents an opportunity for horizontal gene transfer, the main mechanism of evolution in this species and the frequency of co-colonization is reduced in vaccinated children [18]. Vaccination with pneumococcal vaccines is known to affect nasopharyngeal colonization by *S. pneumoniae* either by preventing the acquisition of carriage of a given serotype or by terminating the carriage of the already colonized nasopharynx [18].

Haemophilus influenzae

Haemophilus influenzae is a gram negative bacterium which normally resides in the human nasopharynx. H. influenzae has 6 serotypes (a-f) based on the antigenic properties of the capsular polysaccharide. Non capsular strains of the bacteria are classified as non-typeable. Globally, *H. influenzae* type b (Hib) accounts for 4.1% of severe episodes of pneumonia and about 15% of deaths from pneumonia [16].Other diseases caused by Hib are epiglottitis, septic arthritis, osteomyelitis, and septicemia. Transmission is mediated by respiratory droplets and prevention of the disease is through use of the conjugate capsular polysaccharide-protein vaccine which is especially effective.

Hib conjugate vaccines induce protective humoral immune responses and also reduce circulating strains of Hib in the population by reducing nasopharyngeal carriage of Hib [20]. Vaccine doses are given at 2, 4, 6 months and a booster at 15 months and protect approximately 95% of infants. Universal infant Hib immunization has proven to dramatically reduce Hib invasive disease through direct vaccine protection and an important herd effect related to the reduction in Hib nasopharyngeal carriage in the community [21, 22].

Factors such as immune status of the host, vaccination coverage in the community and bacterial factors tend to determine if colonization with these bacteria results in local respiratory infection or an invasive disease. Nasopharyngeal carriage rates of this bacterium in children vary across regions. A study done (in the post Hib vaccination era) to determine nasopharyngeal carriage of Hib in three distinct geographic areas of France showed the prevalence of Hib in children under 3 years was 1% [23].

A different study in Kenya, determined nasopharyngeal carriage rate of Hib in children under 4 years of age to be 1.7%. The prevalence of carriage did not vary significantly by sex or

location (rural vs. semi-urban) and there was no significant interaction between season, age or sex with carriage prevalence [24].

In the pre-Hib vaccine era, the colonization rate of Hib in infants and children was 3 to 5%. The rate is close to zero in countries that employ widespread Hib vaccination. The reduction of circulating strains in the population results in a herd effect, contributing importantly to the efficacy of the vaccine [25].

Moraxella catarrhalis

Moraxella catarrhalis is a gram negative diplococcus which is a common cause of otitis media in infants and children. It is responsible for 15 – 20% of cases of acute otitis media episodes in infants and children [26]. This bacterium is the leading cause of otitis media with effusion. *M. catarrhalis* DNA is detected in a larger proportion of cases of otitis media with effusion than of acute otitis media [26]. The burden of disease is particularly important as otitis media prone children may develop conductive hearing loss and delayed speech. Likewise, otitis media is the number one reason for which children are prescribed antibiotics in the United States. [27, 28].

High colonization rates (54% - 78%) have been reported in children compared to adults (less than 3%) and colonization rates tend to be higher during winter [29]. What makes *M. catarrhalis* a globally important pathogen is the lack of an efficacious vaccine, the rapid emergence of antibiotic resistance in clinical isolates, and high carriage rates reported in children. In addition, the effectiveness of conjugate vaccines at reducing the incidence of otitis

media caused by *S. pneumoniae* and nontypeable *H. influenzae* suggest that *M. catarrhalis* infections may become even more prevalent. Hence, *M. catarrhalis* is an important and emerging cause of infectious disease for which the development of a vaccine is highly needed.

Staphylococcus aureus

Staphylococcus aureus strains are Gram positive cocci occurring in clusters; strains are implicated in diseases ranging from skin and soft tissue infections to life-threatening sepsis. *S. aureus* is a common cause of infections in children. The advent of drug resistant strains such as the methicillin-resistant *S. aureus* (MRSA) has made treatment of these infections more difficult as little is known about how patterns of colonization with S. aureus in the community have evolved.

Over time, three patterns of carriage can be distinguished. Approximately 20% of individuals almost always carry one type of strain and are called persistent carriers. A large proportion of the population (~60%) harbors *S. aureus* intermittently, and the strains change with varying frequency. Such persons are called intermittent carriers. Finally, a minority of people (~20%) almost never carries *S. aureus* and is called non-carriers. Persistent carriage is more common in children than in adults and it seems to have a protective effect on the acquisition of other strains [31, 32]. A study conducted in Tanzania to determine the nasal carriage of *S. aureus* in under-5 children found a prevalence rate of 40% - of which 11% of this number was MRSA [33].

Factors associated with colonization in children are age (increased colonization among children less than 6 months), recent antibiotic use and vaccination with pneumococcal vaccine which had an inverse relationship [34].

Neisseria meningitidis

Neisseria meningitidis is a Gram negative pathogenic bacterium whose microscopic morphology has a coccoid shape. It is capable of colonizing the nasopharyngeal mucosa without causing disease in the host. *N. meningitidis*, however, can cause meningitis, severe sepsis, septic arthritis and conjunctivitis. Children younger than 5 years are most affected by the disease and rates of infection in sub-saharan Africa can be as high as 1 in 100 children [35]. It has different serotypes which are classified according to the antigenic structure of their polysaccharide capsule. These serotypes have different distributions around the world, with serotype A more prevalent in Africa and Asia but absent in North America. Other serotypes present are B, C, WI35, X and Y.

Bacterial interactions in the nasopharynx

Population dynamics of pathogens in the nasopharynx are based on bacterial interactions which influence which species persist. Interactions may be positive (synergistic) or negative (competitive). These interactions are multifactorial involving both host and bacterial factors and can also be altered by anti-microbial drug or vaccine use [36].

Different mechanisms have been proposed to explain these bacterial interactions. For example, it is thought that production of the bactericidal H_2O_2 by some species (such *S. pneumoniae*) eliminates *S. aureus* or *H. influenzae* even though these bacteria produce catalase [33]. A second interaction mechanism is targeting structures that mediate adherence to the epithelium of the competing microorganism. For example, neuraminidase expressed by the pneumococcus is able to prevent adherence of some strains of *H. influenzae* [37].

Thirdly, the expression of phosphorycholine by *S. pneumoniae and H. influenzae* which mediates bacterial adherence to the host mucosa. Pre-exposure to one of the two species induces the production of antibodies against phosphorylcholine, thereby promoting clearance of the other co-colonizing species [38]. Finally, host immune status also influences bacterial interaction in the nasopharynx. HIV infection in a host has been associated with increased pneumococcal carriage rates compared with non – HIV infected persons [37].

Studies have found a synergist relationship between *S. pneumoniae* and *M. catarrhalis* in healthy children. A negative association was however found between *S. pneumoniae* and *S. aureus* in healthy children [39]. Among children with AOM, competitive associations were found between *Haemophilus influenzae* and *S. pneumoniae* and between *H. influenzae* and *M. catarrhalis*; rates of colonization with *H. influenzae* were higher [39, 40]. However, when *H. influenzae* was present with *M. catarrhalis,* odds of *S. pneumoniae* colonization increased by >2-fold [41].

Several studies discuss the nasopharyngeal bacterial prevalence in healthy children or in children with acute otitis media infections. A common theme for most of these studies is race

and geography as these studies were conducted in Caucasian or Asian children residing in America, Europe or Asia. These undoubtedly give rise to population dynamics that might differ from that of an African child on account of race, geography and full vaccine coverage. There is however limited information available about these bacterial population dynamics in African children, or any other population, and how factors such as a concomitant febrile illness, geography, socioeconomic status and vaccine coverage influence the bacterial nasopharyngeal mileu. A better understanding of these bacterial interactions in the nasopharynx is important for several reasons: Colonization is the initial step in the disease process. Colonized children serve as reservoirs for bacterial transmission to others in the community. Additionally, antimicrobial drugs or vaccines, which target specific bacterial species, may alter polymicrobial interactions in the nasopharynx and have unanticipated consequences [41].

METHODS

Study population

The population for this study was derived from a cross-sectional study of 999 children aged 2 months to 10 years of age. Children in this study were enrolled after presenting at the outpatient clinic of two different hospitals located in two different regions in the United Republic of Tanzania. These two regions were: Dar es Salam which is the large economic capital city of Tanzania and Ifakara which is a small rural town in south central Tanzania.

Enrollment into this study was from April to August 2008 for patients presenting at the outpatient clinic of Dar es Salam and from June to December for those presenting at the outpatient clinic in Ifakara. This study population was recruited to allow for a comprehensive

exploration of the spectrum of possible etiologies of fevers in African children. Having DNA samples from these children also afforded the opportunity of studying nasopharyngeal colonizers in this population.

Research Design and Study procedures

Children <10 years of age presenting with an axillary temperature of greater than or equal to 38^{o}C were enrolled after screening. Inclusion criteria for this study were: 1) first consultation for the present problem; 2) history of fever lasting for one week or less; 3) main complaint was not an injury or trauma and; 4) no report of an antimalarial or antibiotic medicine taken during the previous week.

A written informed consent from the child's caregiver was obtained if child met all inclusion criteria after which a medical history and clinical examination were conducted in a standardized way. Blood and nasopharyngeal samples were taken to perform a wide range of rapid diagnostic tests, serology, blood culture and molecular analyses for pathogens causing acute fever, according to pre-defined algorithms. Children with severe malnutrition and those requiring immediate live saving procedures (according to the WHO Emergency Triage Assessment and Treatment) were excluded from the study.

Clinical assessment

Research clinicians used a standardized case report form to record the medical history and examination findings. This form had all items relevant for a febrile case listed and had to be checked one by one. Items to be checked included the main complaints, symptoms, clinical features or signs, travel history, potential-at-risk contacts and known chronic conditions.

Investigations

All enrolled subjects underwent a set of investigations that included a full blood cell count, test for malaria (rapid test and thick film for plasmodium species), a rapid test for typhoid, liver (ALT) and kidney (creatinine) function tests. Additional tests done were: a cerebrospinal fluid analysis and Gram stain; skin aspiration for Rickettsia; examination of stools for Entamoeba species; stool culture; rapid antigen test for adenovirus and rotavirus, urinalysis and urine culture (Dar es Salaam site only) and chest X-ray. Finally, serologic and polymerase chain reaction (PCR) tests were carried out on blood and nasopharyngeal specimens at a later stage.

Definitive diagnosis

For every case, a definitive diagnosis was established by combining laboratory results and criteria available from international guidelines. On this basis, standardized algorithms were programmed into a computer and assessments were generated automatically from the case report form to ensure consistency and eliminate subjectivity. Designations such as 'severe' and 'systemic infection' were added when a child fulfilled criteria provided in reference WHO guidelines employed in the study. A 'systemic infection' was defined as a febrile illness without any specific localized symptom or sign, due to a parasite (other than malaria), bacteria (other than *Salmonella typhi*) or a virus and documented by serology or PCR on a blood sample.

Laboratory analysis

Specimen collection and storage

Nasopharyngeal (NP) swabs for bacterial colonization were collected from all 999 children enrolled into the study and analyzed for bacterial DNA. These swabs were immediately

stored in 1 ml of STGG transport medium which is a mixture containing skim-milk, tryptone, glucose and glycerol. The STGG vials with NP swabs inside were quickly vortexed and frozen at -70^{o}C until the further analysis.

DNA extraction from nasopharyngeal swabs

All DNA extractions were performed in an access-restricted lab room utilized only for processing clinical samples and under a biological safety cabinet with sterile environment. Frozen NP samples were thawed at room temperature and then vortexed for 15 seconds. Two hundred µl of the sample were added with 100 µl of TE buffer (10mM Tris-HCl, 1 mM EDTA, pH 8.0) containing 0.04 g/ml of lysozyme and 75 U/ml of mutanolysin and then incubated for 1 h in a 37°C in water bath. The subsequent steps were carried out according to Qiagen DNA mini kit protocol. Briefly, 20 µl of proteinase K (600 mAU/ml) and 200 µl of buffer AL were added to the samples and vortexed for 5 seconds, followed by incubation in water bath at 56°C for 30 minutes and then briefly centrifuged to remove drops from inside of the lid.

The tubes were treated with 260 µl of 96% molecular biology grade ethanol, vortexed for 15 seconds and transferred to a QIAamp Spin column and centrifuged at 6000 x g for 1 minute. Eluted liquid was discarded and the Qiagen spin column installed in a new collection tube. The columns were washed with 500 µl of buffer AW1 followed by 500 µl of buffer AW2. After washing, the columns were placed into a sterile 1.5 ml Eppendorf tube, added with 100 µl of elution buffer (AE) and incubated 5 minutes at room temperature. To finally elute DNA, the tubes were centrifuged at 6000 x g for 1 minute and immediately stored at -70°C. DNA from the reference strain TIGR4 was also extracted for positive control and DNA concentrations were obtained by the Nanodrop method (Nanodrop Technologies, Wilmington, DE).

Quantitative PCR

Quantitative PCR (qPCR) assays targeting the following genes *lyt*A, *nuc, hpd, cop*B and *sod*C, carried by all *S. pneumoniae, S. aureus, H. influenzae, M. Catarrhalis,* and *N. meningitidis* respectively, were performed. The qPCR assays utilized the following primers and probes.

To determine the total density of *S. pneumoniae* in CFU/ml**,** pre-optimized concentrations of the forward primer was used to target the lyt A gene (5′-ACGCAATCTAGCAGATGAAGCA-3′; 200 nM), reverse primer (5′-TCGTGCG TTTTAATTCCAGCT-3′; 200 nM), and probe (5′-FAM-TGCCGAAAACGCTTGATACAG GGAG-3-BHQ1; 200 nM). In order to quantify the molecular bacterial load (CFU/ml) purified DNA from *S. pneumoniae* reference strain TIGR4 was diluted serially to prepare standards representing , 4×10^1, 4×10^2, 4×10^3, 4×10^4, 4×10^5 or 4×10^6 CFU.

Total density of *S. aureus* in CFU/ml, was determined using pre-optimized concentrations of the forward primer to target the nuc gene (5′-GTTGCTTAGTGTTAACTTTAGTTGTA-3′; 800 nM), reverse primer (5′-AATGTCGCAGGTTCTTTATGTAATTT-3′; 800 nM), and probe (5-FAM-AAGTCTAAGTAGCTCAGCAAATGCA-3-BHQ1; 400 nM).

The total density of *H. influenzae* in CFU/ml, was determined using primers and probe to target the hpd gene: forward primer (5′-GGTTAAATATGCCGATGGTGTTG-3′; 100 nM), reverse primer (5′-TGCATCTTTACGCACGGTGTA-3′; 300 nM), and probe (5′-HEX-TTGTGTACACTCCGT “T-BHQ1” GGTAAAAGAACTTGCAC-3′; 100 nM).

M. catarrhalis had its total density in CFU/ml also determined by using primers and probe to target the copB gene. Forward primer (5′-CGTGTTGACCGTTTTGACTTT-3′; 200nM), reverse primer (5′-TAGATTAGGTTACCGCTGACG-3′; 200nM), and probe (Cy5-ACCGACATCAACCCAAGCTTTGG -BHQ3-3′; 100nM).

Finally, the total density of *N. meningitidis* in CFU/ml was obtained by using primers to target the sodC gene; forward primer (5′-GCACACTTAGGTGATTTACCTGCAT-3′; 400nM), reverse primer (5′-CCACCCGTGTGGATCATAATAGA-3′; 400nM), and probe (5′-CATGATGGCACAGCAACAAATCCTGTTT-3′; 200nM).

Quantitative PCR reactions were carried out in a final 25 µl volume and performed by use of Platinum qPCR superMix (Invitrogen), according to the instructions of the manufacturer, with 2.5 µl of purified DNA and respective concentrations of each primer and probe set.

A no-template control was always included in every run. To quantify the number of genome copies of pathogens present in each clinical sample, a standard curve using 10 to 10^8 copies of the respective pathogen genome was run in parallel. All these standards were optimized in the Vidal laboratory so standard curves and quantification demonstrated amplification efficiency between 94 and 98% (recommended 90-110%) (D'Haene et al.). All qPCR reactions were run in a CFX96 real time system (BioRad, Hercules, CA) with the following cycling parameters: 95°C for 2 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Negative samples were defined with cycle threshold (CT) values, if any, greater than >35.

Statistical Analysis

An exploratory analysis of variables in the dataset was done. A frequency procedure showed the distribution of the categorical variables such as sex, site, acute respiratory infection, malaria, typhoid etc. Next, a univariate analysis was performed to look at the distribution of age (months) and bacterial pathogen densities (CFU/ml). The Age of enrolled children was categorized into; age ≤12 months, >12-36 months and > 36 months. The densities for each bacterial pathogen was transformed into a categorical variable by dichotomizing bacterial densities into a binary outcome of either low ($1–10^5$ CFU/ml) or high (> 10^5CFU/ml) and absence (0 CFU/ml) or presence (> 0 CFU/ml).

The main outcomes of interest were the relationships of bacteria in children with febrile diseases. Chi-square tests were performed to examine whether there was an association among bacterial densities of all bacteria and between each bacteria and other independent categorical variables in the dataset. This was performed to help in selecting relevant variables for model building. Next, an assessment for multicollinearity between the independent variables was done.

Logistic regression models were used to determine if colonization by one bacterial species was associated with colonization by the other four bacterial species. To examine the effects of covariates on each bacteria species, we modeled colonization by all five bacteria separately. The manual elimination approach and the backward elimination approach were both used to determine bacterial associations and identify other risk factors if present within the sample. Colonization by *S. pneumoniae*, *H. influenzae*, *S. aureus*, *M. catarrhalis* and *N. meningitidis* was modeled separately, and each model included the presence/absence of the other

bacterial species as the main exposures of interest and adjustments for age, sex, site and acute respiratory infection status.

For each of the models built, evaluation of the model was done (test of model fit) using the 'Goodness of fit' tests such as the Hosmer-Lemeshow test. All statistical analysis was performed using SAS version 9.1.

Ethical Approval

This study is a secondary epidemiological study with de-identified subject I.D. information extracted from the primary study and therefore is considered as "non-human subjects research", which should be exempted from IRB submission.

RESULTS

The study population consisted of 513 (51.35%) boys and 486 (48.65%) girls. The age distribution of this study population was; ≤12 months (32%), >12 – 36 months (45%) and >36 months (22%) shown in table 1. Mean age for boys was 24 months (Interquartile range of 23 months) and mean age for girls was 26 months (Interquartile range of 24 months). Among the enrolled children, ~62% of them had an acute respiratory infection, whereas ~10% were diagnosed with malaria, ~3% typhoid, ~6% with urinary tract infection (UTI) and near 13% had a systemic infection when they present themselves to either the rural or urban site. Almost equal proportion of children were enrolled at either rural or urban outpatient clinics. Table 1 shows the characteristics of the study population.

Prevalence of colonization by bacterial pathogens as determined by qPCR

Among the nasopharyngeal swabs collected from the enrolled children, the overall prevalence of presence of colonization by *S. pneumoniae* was 81%, *H. influenzae* (74%), *S. aureus* (23%), *M. catarrhalis* (91%) and *N. meningitidis* (50%), see table 2. After dichotomizing bacterial densities of these pathogens into high or low, the prevalence of colonization by high density *S. pneumoniae, H. influenzae, S. aureus, M. catarrhalis* and *N. meningitidis* was 68.4%, 66%, 7.6%, 83.8% and 14.2% respectively (Table 3).

Our detailed analyses revealed that only 5.2% of the children enrolled into this study were colonized by one species of any bacterial pathogen. Colonization by two or more species of bacteria concurrently was distributed as follows; 12.5% of children were colonized by two bacterial species, 32.5% by three bacterial species, 33.8% by four bacterial species and 13.5% were colonized by five bacterial species (Table 4). Therefore, carriage of more than one potential respiratory pathogen is more common in this particular population. For children colonized concurrently by two bacterial species, the most prevalent combination was *S. pneumonia* and *M. catarrhalis*. Among children colonized with three bacterial species, the most prevalent combination was *S. pneumoniae, H .influenzae and M. catarrhalis.* Children colonized concurrently by four bacterial species had a predominant combination of *S. pneumoniae, H. influenzae, M. catarrhalis* and *N. meningitidis.* Importantly, ~65% children carried *S. pneumoniae and M. catarrhalis* in their nasopharynx at the same time. Table 4 shows the distribution of bacterial types in the study population.

For children with an acute respiratory infection, the overall prevalence of the bacterial species were; *S. pneumoniae* (84%), *H. influenzae* (79%), *S. aureus* (22%), *M. catarrhalis* (92%) and *N.*

meningitidis (50%) respectively. Table 5 shows the overall prevalence of these bacterial species in children with different febrile diseases.

Tests for associations of bacterial densities of all bacteria species showed associations between some bacterial groups.

Correlation of bacterial densities

The Spearman's correlation coefficients for presence of bacteria (density > 0 CFU/ml) between *S .pneumoniae* and *H. influenzae* was 0.31 (p value <0.0001), that between *S. pneumoniae* and *M. catarrhalis* was 0.37 (p value <0.0001) and was 0.31 (p value <0.0001) for that between *H. influenzae* and *M. catarrhalis*. Negative correlation coefficients were obtained between *S. aureus* and *S. pneumoniae*, -0.07 (p value 0.002) and between *S. aureus* and *H. influenzae*, -0.07 (p value 0.04). Results also showed weak correlations of presence of bacterial densities for *N. meningitidis* with the other bacterial species when assessing by the Spearman's correlation method.

Associations between colonization by the five bacterial respiratory pathogens using logistic regression

Logistic regression models of bacterial colonization densities (absence or presence) are displayed in Table 6. The model of colonization by *S. pneumoniae* showed a positive association with colonization by *H. influenzae* (OR 2.21, 95% CI 1.47-3.3.30, p-value <0.0001) and colonization by *M. catarrhalis* (OR 5.63, 95% CI 3.27- 9.70, p-value <0.0001), but was negatively associated with colonization by *S. aureus* (OR 0.56, 95% CI 0.36-0.85, p-value <0.0001), when controlling for age, sex, site and acute respiratory infection status. The site of a child's enrollment was found

to be associated with colonization by *S. pneumoniae* (OR O.28, 95% CI 0.18-0.44, p-value <0.0001). This meant that the odds of being colonized by *S. pneumoniae* in a child enrolled at the urban clinic is about 0.3 times less than that of a child enrolled at the rural clinic. The age of the child (>36 months) at enrolment was associated with colonization by *S. pneumoniae* (OR 0.62, CI 0.38-1.00, p-value 0.049), meaning that children older than 36 months had lower odds of colonization when compared to children ≤12 months.

The model of colonization by *H. influenzae* also showed a positive association with colonization by *S. pneumoniae* (OR 2.18, 95% CI 1.46-3.26, p-value <0.0001) and *M. catarrhalis* (OR 3.76 95% CI 2.17-6.51, p-value <0.0001) while controlling for age, sex, site and acute respiratory infection status. We also find a positive association between presence of colonization by *H. influenzae* and colonization by *N. meningitidis* (OR 1.43, 95% CI 1.01-2.03, p value <0.043) and a negative association involving colonization by *H. influenzae* and *S. aureus* (OR 0.63, 95% CI 0.43-0.92, p-value 0.017). The site where the child was enrolled was also associated with colonization by *H. influenzae* (OR 0.39, CI 0.27-0.57, p-value <0.0001). Also meaning that the odds of being colonized by *H. influenzae* for children enrolled at the urban clinic was 0.4 times less than that for children enrolled at the rural clinic. Presence of an acute respiratory infection was associated with colonization *H. influenzae* (OR 1.46, CI 1.04-2.05, p-value 0.03).

Logistic regression also showed that colonization by *S. aureus* was found to be associated positively with colonization by *N. meningitidis* (OR 1.74, 95% CI 1.25-2.43, p-value 0.01) and negatively associated with colonization by *S. pneumoniae* (OR 0.52,95%CI 0.34-0.77, p-value 0.0013) while controlling for age, sex, site acute respiratory infection status.

Positive associations were also found in the model for colonization by *M. catarrhalis* with colonization by *S. pneumoniae* (OR 5.09, 95% CI 2.93-8.84, p-value <0.0001) and *H. influenzae* (OR 3.65, 95% CI 2.09-6.39, p-value <0.0001) while controlling for age, sex, site and acute respiratory infection status. There was also a positive association between colonization by *M. catarrhalis* and *N. meningitidis* (OR 1.99, 95% CI 1.12-3.54, p-value 0.018). Also the site where the child was enrolled was associated with colonization by *M. catarrhalis* (OR 0.11, 95%CI 0.04-0.28, p-value <0.0001), showing that the odds for colonization by *M. catarrhalis* in urban children was 0.1 times less than those of rural children.

Finally, the model for colonization by high density *N. meningitidis* was positively associated with colonization by *S. aureus* (OR 1.73, 95% CI 1.24-2.41, p-value 0.0012) and *M. catarrhalis* (OR 2.23, 95% CI 1.29-3.87, p-value 0.0043) while controlling for age, sex, site and acute respiratory infection status. Colonization by *N. meningitidis* was also positively associated with colonization by *H. influenzae* (1.49, 95% CI 1.05-2.11, p-value 0.024). There was a positive association between the age of child at enrollment and colonization by *N. meningitidis.* This was significant for age groups 13 – 36 months (OR 3.78, 95% CI 2.73-5.23, p-value <0.0001) and age groups > 36 months (OR 3.06, 95% CI 2.09 – 4.48, p-value 0.0001). This meant that the odds of being colonized by *N. meningitidis* was approximately 3 times higher in children aged 13 months, or older, than children 12 months or younger.

DISCUSSION

This study has shown that nasopharyngeal colonization *by S. pneumoniae*, *H. influenzae* and *M. catarrhalis* is common among Tanzanian children with febrile illnesses. In fact,

colonization by *M. catarrhalis* was 91% making it the most prevalent pathogen in the nasopharynx of these children. This high prevalence of *M. catarrhalis* is similar to results presented in an epidemiological study conducted among Australian Aboriginal children [44]. Concurrent nasopharyngeal colonization in a child by four species of bacteria namely; *S. pneumoniae*, *H. influenzae, M. catarrhalis* and *N. meningitidis* was the most prevalent bacterial combination accounting for approximately 34% of the study population (Table 4).

Regression analysis revealed that gender and presence of diseases such as malaria, pneumonia, urinary tract infection and typhoid were not associated with colonization by any of the five bacterial species examined using qPCR results. However, the presence of an acute respiration infection in a child was positively associated with colonization by *H. influenzae* (OR 1.46, CI 1.04-2.05, p-value 0.03). Also, the age of the child and site of enrollment had significant associations with colonization by some bacterial species. These results show that age of the child at enrollment was significantly associated with colonization by *S. pneumoniae* and *N. meningitidis* (Table 4) while site of child enrollment was significantly associated with colonization by all bacterial species examined except for *N. meningitidis* (Table 6). For the 4 bacterial species associated with the site of child enrollment, results showed lower odds of colonization among children enrolled at the urban clinic compared to children enrolled at the rural clinic.

Strong relationships among these five bacterial species were seen from results of this study. The significant negative association between colonization by *S. pneumoniae* and colonization by *S. aureus* is consistent with results of another study conducted in healthy

children [43]. This might be explained by the hydrogen peroxide mediated bactericidal activity of *S. pneumoniae* toward *S. aureus* [43].

Other significant positive associations observed were between colonization by *S. pneumoniae* and *H. influenzae* and *M. catarrhalis*. There was also a positive correlation of the densities of these three pathogens in the nasopharynx of these children. A recent study has shown that the positive association between *S. pneumoniae* and *H .influenzae* might be due to the increased pneumococcal biofilm formation in vivo and in vitro [44]. However, another study did not report a positive association between co-colonization by *S .pneumoniae* and *H. influenzae*. This study described a competitive interaction between these two pathogens believed to be mediated by the host's complements and neutrophils. It was suggested that *H. influenzae* cellular components activate the host innate immune response, thus killing *S. pneumoniae* [46].

Some studies have also confirmed the positive association between *S. pneumoniae* and *M. catarrhalis*. It is believed that *M. catarrhalis* produces a Beta-lactamase enzyme which protects *S. pneumoniae* from certain Beta-lactam antibiotics thereby encouraging its growth [45].

Despite the consistencies in results between this study and other published studies, there are still some limitations to this study. Firstly, the observed associations between bacterial species in this study might be due to potential host confounders which were not controlled for. There was no data about factors such as a child's HIV status, immunization status, breastfeeding status/duration among infants which might have confounded results.

Secondly, the study results might have been influenced by external factors such as; the season/time of year nasopharyngeal samples were collected from enrolled children. These

children were not all enrolled at the same time – enrollment at the urban site began in April and ended in August while that of the rural site was from June to December of the same year. Other external factors that could influence the study results were; exposure to indoor air pollution from firewood smoke which is common among rural African communities and the number of siblings each enrolled child was exposed to.

Finally we did not identify a risk factor (e.g. presence of febrile diseases like malaria, typhoid, pneumonia, gastrointestinal disease or skin diseases) that had a significant association with colonization by any of the bacterial pathogens studied. This might be due to the fact that some of these diseases are caused by organisms such as protozoa, viruses or bacteria other than the five bacterial pathogens studied.

Regardless of these limitations, results from this study have public health implications. This study's results and many other study results will help to answer the questions about whether disease can be prevented by eradicating nasopharyngeal colonization. Surely a good understanding of bacterial interactions in the nasopharynx is required for designing preventive measures such as vaccines and antimicrobial drugs. Also, such knowledge might be useful in designing interventions aimed at reducing the risk of colonization by *S. aureus* especially in this era of growing methicillin-resistant *S. aureus*. A study by Moyo et al detected a 10.5% prevalence of MRSA in healthy Tanzanian children [33]. A recent study found a 50% carriage rate for *M. catarrhalis* amongst HIV infected Tanzanian infants 6 weeks of age [49]. This is troubling as *M. catarrhalis* is a common cause of acute otitis media in infants [26]. Giving this prevalence and the potential for otitis media and subsequent complication of hearing deafness in

a child if left untreated, it is necessary to design interventions to prevent colonization of this pathogen in children.

So far, most studies have sought to understand bacterial interactions among non-African children- a population that might yield results that are different from that of an African child. It is therefore necessary that more studies to understand the bacterial population dynamics of respiratory pathogens should be encouraged in African children as such studies will help produce the facts needed to design interventions tailored to the African child.

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TABLES

Table 1: Characteristics of study participants (N = 999)

Variable	No.	Percent
Sex		
Males	513	51.35%
Females	486	48.65%
Age		
≤ 12 months	324	32.43%
> 12 -36 months	451	45.15%

> 36 months	224	22.42%**Site**
Rural	497	49.75%
Urban	502	50.25%
Acute respiratory infection at time of nasopharyngeal sample collection		
Yes	622	62.26%
No	377	37.74%
Malaria at time of nasopharyngeal sample collection		
Yes	105	10.51%
No	894	89.49%
Typhoid at time of nasopharyngeal sample collection		
Yes	37	3.70%
No	962	96.30%
Urinary tract infection at time of nasopharyngeal sample collection		
Yes	59	5.91%
No	940	94.09%
Systemic infection at time of nasopharyngeal sample collection		
Yes	127	12.71%
No	872	87.29%

Table 2: Showing overall prevalence rates of each bacterial specie in study population

	Overall prevalence rate of each bacteria specie in study population (N=999)				
	S. pneumoniae (%)	*H. influenzae (%)*	*S. aureus (%)*	*M. catarrhalis (%)*	*N. meningitidis (%)*
Prevalence	80.8%	74.2%	23.3%	90.9%	50.1%

Table 3: Distribution of bacteria on nasopharyngeal swabs taken from all children enrolled in the study

Bacterial	**Percentage of each bacteria specie in a given density category (N=999)**

density (CFU/ml)					
	S. pneumoniae (%)	***H. influenzae (%)***	***S. aureus (%)***	***M. catarrhalis (%)***	***N. meningitidis (%)***
Low >0 -10^5	12.4%	8.2%	15.7%	7.1%	35.9%
High >10^5	68.4%	66.0%	7.6%	83.8%	14.2%
0	19.2%	25.8%	76.7%	9.1%	49.9%

Table 4: Distribution of bacterial types in nasopharyngeal swabs tested for all five bacteria using q PCR (number and percentage of total population are also displayed)

No bacterial species identified, 24 (2.4%)		
One bacteria species identified, 52 (5.2%)		
Most common	*M. catarrhalis*	23 (2.3%)
Least common	*H.influenzae*	6 (0.6%)
Two bacterial species identified, 125 (12.51%)		
Most common	*S.pneumoniae + M.catarrhalis*	50 (5.0%)
Least common	*S.pneumoniae + S.aureus*	2 (0.2%)
Three bacterial species identified, 325 (32.53%)		
Most common	*S.pneumoniae + H.influenzae + M.catarrhalis*	218 (21.82%)
Least common	*S.pneumoniae + S.aureus + N.meningitidis*	2 (0.2%)
Four bacterial species identified, 338 (33.83%)		
Most common	*S.pneumoniae + H.influenzae + M.catarrhalis + N.meningitidis*	240 (24.02%)
Least common	*S.pneumoniae + H.influenzae + S.aureus + N.meningitidis*	2 (0.2%)
All five bacterial species identified, 135 (13.51%)		

Table 5: Prevalence of bacterial types in the various febrile diseases among the study population

Presence of bacteria	Acute respiratory infection	Typhoid	Malaria	Clinical Pneumonia	Gastrointestinal disease	Urinary tract infection	Systemic illness
S. pneumoniae	524 (84.2%)	29 (78.4%)	87 (82.9%)	131 (87.3%)	82 (79.6%)	50 (84.8%)	100 (78.7%)
H. influenzae	490 (78.8%)	31 (83.8%)	73 (69.5%)	123 (82.0%)	79 (77.5%)	37 (62.7%)	94 (74.0%)
S. aureus	124 (21.7%)	7 (23.3%)	18 (19.2%)	27 (19.6%)	19 (20.2%)	16 (29.6%)	25 (22.1%)
M. catarrhalis	575 (92.4%)	34 (91.9%)	100 (95.2%)	144 (96.0%)	92 (90.2%)	53 (89.8%)	111 (87.4%)
N. meningitidis	285 (49.8%)	21 (70.0%)	45 (47.9%)	60 (43.5%)	42 (44.5%)	26 (48.2%)	53 (46.9%)

Table 6: Association between different bacterial species adjusting for age, sex, site and acute respiratory infection.

	OR (95% CI) for colonization with selected bacteria				
	S. pneumoniae	***H. influenzae***	***S. aureus***	***M. catarrhalis***	***N. meningitidis***
S. pneumoniae					
Absence (ref)					
(0 CFU/ml)	--------	2.18(1.46-3.26)	0.52(0.34-0.77)	5.09(2.93-8.84)	---------
H. influenzae					
Absence (ref)					
(0 CFU/ml)	2.21 (1.47-3.30)	---------	---------	3.65(2.09-6.39)	1.49(1.05-2.11)
S. aureus					
Absence (ref)					
(0 CFU/ml)	0.56 (0.36-0.85)	0.63(0.43-0.92)	---------	---------	1.73(1.24-2.41)
M. catarrhalis					
Absence (ref)					
(0 CFU/ml)	5.63 (3.27-9.70)	3.76(2.17-6.51)	---------	---------	2.23(1.29-3.87)
N. meningitidis					
Absence (ref)					
(0 CFU/ml)	-------	1.43(1.01-2.03)	1.74(1.25-2.43)	1.99(1.12-3.54)	---------
Sex					
Male (ref)	0.84(0.57-1.23)	0.91(0.65-1.26)	0.85(0.62-1.17)	1.01(0.59-1.71)	0.91(0.69-1.21)
Site					
Rural (ref)	0.28(0.18-0.44)	0.39(0.27-0.57)	0.61(0.43-0.86)	0.11(0.04-0.28)	1.04(0.76-1.41)
Age					
≤ 12 months (ref)					
> 12 -36 months	1.24(0.78-1.93)	1.39(0.97-1.99)	0.92(0.63-1.35)	1.34(0.70-2.57)	3.78(2.73-5.23)
>36 months	0.62(0.38-1.00)	1.00(0.66-1.51)	1.01(0.65-1.55)	1.40(0.72-2.74)	3.06(2.09-4.48)
Acute respiratory infection					
Absence (ref)	1.15(0.78-1.69)	1.46(1.04-2.05)	0.74(0.53-1.03)	0.88(0.51-1.49)	0.97(0.72-1.30)

OR, odds ratio; CI, confidence interval. Significant ORs and 95% CI are underlined. These models adjusted for age, sex, site and acute respiratory infection status.