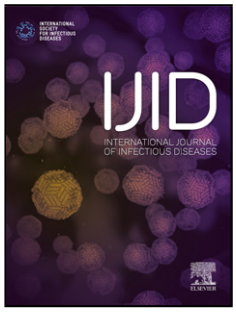

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Carriage of penicillin non-susceptible pneumococci among children in northern Tanzania in the 13-valent pneumococcal vaccine era

Running title: Colonization pneumococci, Tanzania

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Highlights

- Pneumococcal carriage among the sampled children was 31 % (244/775)
- Penicillin non-susceptibility increased during 2013-2015 after PCV13 introduction

- Non-susceptibility to amoxicillin/ampicillin and ceftriaxone was low
- The prevalence of PCV13 serotypes decreased from 56 % in 2013 to 23 % in 2015
- The majority of the children had been treated with antibiotics in the past 3 months

Abstract

Objectives

To determine antibiotic susceptibility and serotype distribution of colonizing *Streptococcus pneumoniae* in Tanzanian children, we performed serial cross-sectional surveys following national introduction of the 13-valent pneumococcal conjugate vaccine PCV13 in December 2012.

Methods

775 children below 2 years of age were recruited and sampled from nasopharynx at primary health centres in Moshi, Tanzania between 2013 and 2015. *S. pneumoniae* were isolated by culture and tested for antibiotic susceptibility by disc-diffusion and E-tests; molecular testing was used to determine serotype/group.

Results

Penicillin non-susceptibility in the isolated pneumococci increased significantly from 31 % (36/116) in 2013, to 47 % (30/64) in 2014 and 53 % (32/60) in 2015. Non-susceptibility to amoxicillin/ampicillin and ceftriaxone was low (*n*=8 and *n*=9, respectively), while 97 % (236/244) of the isolates were non-susceptible to trimethoprim-sulfamethoxazole. The majority of the children (54 %, *n*=418) had been treated with antibiotics in the past 3 months and amoxicillin/ampicillin was overall the most commonly used antibiotics. Carriage of penicillin non-susceptible pneumococci was more common in children with many siblings. The prevalence of PCV13 serotypes among the detected serotypes/groups decreased from 56 % (40/71) in 2013 to 23 % (13/56) in 2015.

Conclusions

Penicillin non-susceptibility in *S. pneumoniae* colonizing Tanzanian children increased during an observation period shortly after the PCV13 introduction. Measures to ensure rational use of antibiotics and more effective systems for surveillance of antibiotic resistance and serotype distribution are needed to assure continued effectual treatment of pneumococcal disease.

List of abbreviations

CI: confidence interval; EUCAST: European committee on antimicrobial susceptibility testing; HIV: human immunodeficiency virus; KCMC: Kilimanjaro Christian Medical Centre; MIC: minimum inhibitory concentration; OR: odds ratio; PCV: pneumococcal conjugate vaccine; PCV7: 7-valent pneumococcal conjugate vaccine; PCV13: 13-valent pneumococcal conjugate vaccine; RTI: respiratory tract infection.

Keywords

Streptococcus pneumoniae; Drug resistance, Bacterial; Air pollution; Pneumococcal vaccines; Nasopharyngeal colonization.

Background

Streptococcus pneumoniae, or the pneumococcus, is still in the era of pneumococcal conjugate vaccines (PCVs) an important cause of bacterial pneumonia, sepsis and meningitis in children (Wahl, O'Brien et al. 2018). Nasopharyngeal colonization of pneumococci is most common in pre-school children; it precedes infection and enables horizontal spread of the bacteria (Bogaert, De Groot et al. 2004). Children in low-income countries have the highest burden of colonization, which may be due to higher exposure to risk factors such as crowding (Abdullahi, Karani et al. 2012).

High antibiotic use is associated with increased risk of nasopharyngeal carriage of penicillin non-

susceptible pneumococci in children (Arason, Kristinsson et al. 1996; Kobayashi, Conklin et al. 2017; Melander, Molstad et al. 1998). According to the WHO Integrated Management of Childhood Illness oral amoxicillin is the drug of choice for treatment of childhood pneumonia at primary health care level (WHO 2014b). Children aged 2 months – 5 years presenting with fast breathing or chest indrawings, not improving after treatment with bronchodilator in the case of wheezing, are assumed to suffer from pneumonia and are eligible for antibiotic treatment (WHO 2014b). An earlier study at paediatric health care facilities in Moshi, Tanzania, showed remarkably high prescription of antibiotics to children presenting with symptoms such as cough, but no signs of pneumonia (Gwimile, Shekalaghe et al. 2012).

The pneumococcal conjugate vaccine (PCV) was developed to generate an appropriate immune response in small children and so defend against the 7, 10 or 13 of the most disease-causing pneumococcal serotypes. In December 2012, the 13-valent pneumococcal conjugate vaccine (PCV13) was introduced into the national immunization program in Tanzania, and was given to children at 4, 8 and 12 weeks of age with no catch-up campaign at the time of enrolment (IVAC 2012). The PCV13 has proved to dramatically reduce the incidence of invasive pneumococcal disease in children from the included serotypes (von Gottberg, de Gouveia et al. 2014; Cutts, Zaman et al. 2005) and to lower the incidence of radiologically proven pneumonia in South Africa and the Gambia (Klugman, Madhi et al. 2003; Cutts, Zaman et al. 2005, Mackenzie, Hill et al. 2017). Soon after introduction of the PCV10 in Kilifi, Kenya carriage of vaccine serotypes in children under 5 years of age decreased by 64 %, also proving herd-effect in older children and adults (Hammitt, Akech et al. 2014). The proportional impact of the vaccine depends on serotype distribution prior to introduction of the vaccine. A study on healthy children in Dar es Salaam showed 63 % coverage of PCV13 in colonizing pneumococci pre-vaccination (Moyo, Steinbakk et al. 2012).

Both the 7- and 13-valent pneumococcal conjugate vaccines have been shown to reduce antibiotic-

resistant invasive pneumococcal disease in countries such as South Africa, the US and Canada (Klugman, Madhi et al. 2003; Tomczyk, Lynfield et al. 2016; Tyrrell, Lovgren et al. 2009). However, the effect of PCV13 on antibiotic non-susceptibility in colonizing and/or invasive pneumococci is, in large parts of sub-Saharan Africa less known due to lack of routine culture and antibiotic susceptibility testing (Balsells, Guillot et al. 2017; Hackel, Lascols et al. 2013; Williams, Isaacs et al. 2018).

Household air pollution due to incomplete combustion of solid fuels during cooking, heating or lighting has emerged as an important risk factor for childhood pneumonia (GBD 2015 LRI Collaborators 2017; Gordon, Bruce et al. 2014). Exposure to the toxic pollutants, such as particulate matter, has proved to affect the defence against microorganisms on all levels of the respiratory tract (Barregard, Sallsten et al. 2008; Gordon, Bruce et al. 2014; Hawley and Volckens 2013; Rylance, Fullerton et al. 2015; Zhou and Kobzik 2007). Although less studied, household air pollution may also alter the colonizing microbes of the respiratory tract, including pneumococci, which may increase the risk of infection (Hussey, Purves et al. 2017; Rylance, Kankwatira et al. 2016).

This study aimed to determine antibiotic susceptibility and serotype/serogroup distribution of colonizing pneumococci in Tanzanian children during a three-year period shortly after the introduction of the PCV13 in Moshi, Tanzania. We further aimed to assess the epidemiology of pneumococcal carriage including its relation to household air pollution and antibiotic use.

Methods

Recruitment of children

Serial cross-sectional surveys were performed in October-November 2013, February-March 2014 and February-April 2015 in Moshi urban district, in the Kilimanjaro Region of northern Tanzania. In 2012, Moshi urban district had an estimated population of over 180,000 people (National Bureau of Statistics, Ministry of Finance 2013). The district had a total of 24 public health facilities; four hospitals, five health centres and 15 dispensaries. The samples were collected at six different health

facilities, chosen to represent different geographical locations within the wider district. These included both dispensaries (Bondeni, Njoro, Rau) and health centres (Pasua, Majengo, Shirimatunda) (Additional file 1). All children below two years of age attending study health facilities with their parent or guardian for medical attention including routine growth monitoring were invited to participate in the study. The parents or guardians responded to a questionnaire for socio-demographic information and health status of the child. Parents or guardians also responded to questions on antibiotic use; they were asked to give the name of the antibiotic used in order to avoid confusion with other medicines. If the patient's medical log was brought to the clinic, it was reviewed to compliment the parent/guardian's response. To determine air pollution on household level, parents or guardians participating in 2015 were asked additional questions such as use of stoves, cooking location and construction of the household building. Current weight and length of the child were recorded. Due to greater numbers of patients attending health centres in areas attributed by higher population density, a larger proportion of children per study year were sampled at Pasua and Majengo.

Specimen collection

A nasopharyngeal sample was collected from each child according to standard procedure (Satzke, Turner et al. 2013), using a Blue-cap E-swab (Copan Diagnostics Inc., Murrieta, USA). The samples were stored in a cool box and were brought to the Clinical Laboratory at Kilimanjaro Christian Medical Centre (KCMC), Moshi, Tanzania, for culture, all within six hours.

Isolation and identification of *S. pneumoniae*

At the KCMC laboratory, the samples were inoculated on goat blood agar (HiMedia, Mumbai, India) that were incubated at 37°C in sealed containers (Oxoid Limited, Hampshire, UK) along with a CO_2 gas pack (BD GasPak^{TM} EZ CO_2 Container System) and a CO_2 indicator (BD CO_2 Indicator 0,5 mL) for 16-20 hours, and a further 40-44 hours if no pneumococci were found at the first examination. Identification of *S. pneumoniae* was based on colony morphology and optochin sensitivity (≥14 mm).

All pneumococcal isolates were stored in STGG medium at -20°C at KCMC laboratory and transported frozen to the Department of Infectious Diseases at Gothenburg University, Sweden.

Antimicrobial susceptibility testing

Antibiotic susceptibility testing was performed at the KCMC laboratory and determined by disc-diffusion and E-tests according to the methods and breakpoints published by the European Committee on Antimicrobial Susceptibility Testing (EUCAST 2018). The tests were performed on Mueller-Hinton agar (Oxoid), supplied with 5 % added goat blood and 20 mg/L β-NAD (Applichem, Darmstadt, Germany). Inoculated plates were incubated at 37°C in CO_2 environment as described above. The following antimicrobial discs were used: oxacillin (screening disc for beta-lactam resistance) (1 µg), trimethoprim-sulfamethoxazole (1.25/23.75 µg), erythromycin (15 µg), clindamycin (2 µg), norfloxacin (screening disc for fluoroquinolone resistance i.e. levofloxacin and moxifloxacin) (10 µg) and tetracycline (30 µg) (all from Oxoid). If the oxacillin disk clearance zone was less than 20 mm, minimal inhibitory concentration (MIC) was determined by E-tests for penicillin G, ampicillin and ceftriaxone (each at 0.016-256 µg/mL, BioMérieux, Marcy l'Etoile, France). Resistant or intermediate isolates were all referred to as non-susceptible. Multi-drug resistance was defined as non-susceptibility against three or more classes of antimicrobial agents including the beta-lactams (i.e. penicillin G, ampicillin or ceftriaxone) (Finkelstein, Huang et al. 2003; Moyo, Steinbakk et al. 2012).

Nucleic acid extraction and molecular characterization of the strains

Almost all pneumococcal isolates were found to be non-viable after storage and transport to Gothenburg. Further analyses were therefore performed by molecular methods for confirmation of species identification, and for determination of serotypes/serogroups. DNA was extracted from 100 µL of STGG storage medium containing the bacterial isolate diluted in 900 µL of phosphate buffered saline (PBS) using the MagNA Pure LC instrument (Roche Diagnostics, Mannheim, Germany) and

the Total Nucleic Acid Large Volume Kit (Roche Diagnostics) The extracted nucleic acids were eluted in 100 µL elution buffer and stored at -20°C awaiting further analysis.

S. pneumoniae identification was performed by detection of the pneumococcal capsule coding gene *cpsA* by using real-time qPCR (preprint available at https://www.biorxiv.org/content/early/2018/09/12/415422). A Cycle threshold (Ct) value <40 was considered a positive result. Samples that were negative for the capsule gene were further tested for presence of the "Xisco" gene, shown to be a unique marker for identification of *S. pneumoniae* (Salva-Serra, Connolly et al. 2018). In order to verify the presence of DNA in the "Xisco" gene negative samples, 16S analysis was performed according to Hauben *et al.* (Hauben, Vauterin et al., 1997). A flow chart of the analyses performed is shown in Figure 1.

Serotyping of *S. pneumoniae*

Initially, detection of 40 different serotypes was performed using a multiplex real-time PCR protocol published by Centers for Disease Control and Prevention (CDC) with slight modifications as previously described (Birindwa, Emgard et al. 2018). For *cpsA* positive samples in which the serotype could not be detected by the multiplex PCR, the serotypes/serogroups were determined using a modified Sequetyping protocol (Birindwa, Emgard et al. 2018). Briefly, two PCR reactions were set up to amplify the whole *cpsB* gene. The PCR products were sent to GATC Biotech (Cologne, Germany) for purification and sequencing using the four PCR primers. The 1006 bp sequence product was matched to a reference database for determination of the serotype/serogroup.

Differentiation of serotype 6 was performed using a DNA sequencing-dependent approach (preprint available at https://www.biorxiv.org/content/early/2018/09/12/415422). A single nucleotide polymorphism (SNP) that distinguishes serotypes 6A/6C (guanine) and serotypes 6B/6D (adenine) in sequence nucleotide position 584 of the *wciP* region was detected. Subsequently, for differentiation of

serotype 6A and 6C a 6 bp deletion in the *wzy* gene was detected. Differentiation of serotype 6B and 6D was done by a single PCR analysis.

Those samples that did not carry the pneumococcal capsule genes *cpsA* and *cpsB*, but were positive for the "Xisco" gene, thus being non-encapsulated *S. pneumoniae*, were further analysed for the presence of the genes *aliC* and *aliD*. These genes are described as identification markers for non-typeable pneumococci according to Park *et al.* (Park, Kim et al. 2012).

Statistics

Univariable and multivariable logistic regression were performed for determination of risk factors for pneumococcal carriage and for carriage of penicillin non-susceptible pneumococci in all included children. To determine whether changes in penicillin non-susceptibility were significant between the years, a multivariable logistic regression was performed for all carrier positive children. Risk factors described in former studies were considered covariates and were adjusted for in all multivariable models (Abdullahi, Karani et al. 2012; Arason, Kristinsson et al. 1996; Bogaert, De Groot et al. 2004; Kobayashi, Conklin et al. 2017; Melander, Molstad et al. 1998). The analyses were made using IBM SPSS Statistics v. 25, the significance of coefficients was tested for using Wald's test; *p*-values ≤0.05 were considered significant.

Results

Characteristics of the study population

Eight hundred parents or guardians were informed about the study, 23 of them refused to let their child participate and two were excluded because of young age of the parent or guardian (<18 years) leaving a total of 775 included children. Median age of the children was 8 months (range 0-24 months). Socioeconomic and health information about the children are shown in Table 1. The majority (73 %, *n*=562) were visiting the health facility for vaccination or routine growth monitoring whilst 23 %

(*n*=176) of the children were ill and were brought to be seen by a clinician. In total 78 % (608/775) of the children had received at least one dose of PCV13.

Antibiotic use in the children was high, 54 % (*n*=418) of the children had been treated with antibiotics in the past three months. Most antibiotics (87 %, 131/150) consumed within seven days prior to sampling were prescribed by a clinician (Table 1). The most common reason for antibiotic use in the week preceding sampling was respiratory tract infection (76 %, 77/101, data from 2014-2015). However, only one third (*n*=26) of the antibiotic treated children were reported to have had signs of fast or laboured breathing whilst two thirds (*n*=51) presented with cough and/or runny nose with or without fever according to the parent or guardian. Amoxicillin/ampicillin was the most commonly used antibiotic (Additional file 2).

The majority (85 %, *n*=658) of households included during the period 2013-2015 used solid fuel, i.e. firewood or charcoal, for cooking (Table 1). Most households included in 2015 (88 %, 188/213) reported to use more than one stove (Additional file 3: Table S1). The most commonly used stove was an improved charcoal stove, owned by a total of 165 (77 %) of the households, whilst 27 (13 %) relied mostly on a three-stone fire. About half of the households performed some cooking in the main living area (49 %, *n*=104) or outside (53 %, *n*=112). Kerosene or gas was predominantly used inside whilst charcoal was used both inside and outside (Additional file 3: Table S2). Most household buildings (75 %, *n*=160) were made of a material with high thermal mass such as adobe, baked bricks or concrete (Additional file 3: Table S1).

Nasopharyngeal carriage of *S. pneumoniae* in association to risk factors

The pneumococcal carriage rate among the sampled children was 31 % (244/775), as determined by optochin-susceptibility of isolated pneumococci at the KCMC laboratory in Tanzania. Both univariable and multivariable analyses showed that pneumococcal carriage increased significantly

with age and during respiratory tract infection (Table 2). Neither type of fuel used for cooking, construction of the roof, nor material used to construct the walls showed any relation to carriage of *S. pneumoniae* in the 2015 cohort (Additional file 3: Table S3).

Antimicrobial resistance patterns of *S. pneumoniae*

The antimicrobial susceptibility pattern was determined at the KCMC laboratory for the 244 pneumococcal isolates (Table 4). Almost all isolates were non-susceptible to trimethoprim-sulfamethoxazole (97 %, *n*=236). Nearly half of the pneumococcal isolates were resistant to oxacillin (46 %, *n*=112) and thus considered resistant to penicillin V (EUCAST 2018). Of the 112 isolates resistant to oxacillin, 108 were further analysed with MIC determination for penicillin G, ampicillin and ceftriaxone. Whereas none of the isolates were found to be resistant to penicillin G (MIC >2 mg/L), 98 isolates were intermediate (MIC >0.06-2 mg/L), as determined by using EUCASTs breakpoints for pneumococcal infections other than meningitis (EUCAST 2018). Thus, in total 41 % (98/240) of the isolates had MIC values greater than 0.06 mg/L for penicillin G and were therefore considered penicillin non-susceptible pneumococci. Few isolates were intermediate to ampicillin (*n*=8, MIC >0.5-2 mg/L) or ceftriaxone (*n*=9, MIC >0.5-2 mg/L), and none were resistant. Penicillin non-susceptibility increased significantly during the years studied from 31 % (36/116) in 2013, to 47 % (30/64) in 2014 and 523% (32/60) in 2015 (Figure 2). Multi-drug resistance (non-susceptibility towards at least three or more classes of antimicrobial agents) was overall 23 % (56/244) (Figure 2).

Risk factors for carriage of penicillin non-susceptible pneumococci

Having more siblings was found to be a risk factor for colonization of pneumococci with reduced susceptibility to penicillin in the univariable and multivariable analyses (Table 3). Girls were more commonly colonized with penicillin non-susceptible pneumococci as shown in the multivariable analysis (Table 3).

Molecular identification of *S. pneumoniae*

Of the 244 isolates identified as *S. pneumoniae* by optochin-susceptibility testing at the KCMC laboratory, 241 were further analysed by biomolecular testing in Gothenburg, Sweden (Figure 1). Two hundred and five (85%) isolates were confirmed to be *S. pneumoniae* by the presence of the *cpsA* gene. Among the 36 isolates that were negative for the *cpsA* gene, 11 were further confirmed as non-capsulated or non-typeable *S. pneumoniae* by the presence of the "Xisco" and the *aliC/aliD* genes. In the remaining 25 samples, no PCR amplification could be achieved, possibly due to degradation of bacterial DNA during storage and transport.

Serotype distribution

The serotypes or serogroups were determined in 175/205 of the pneumococcal isolates analysed by multiplex real-time PCR or by the modified Sequetyping protocol, both performed in Gothenburg, Sweden (Figure 1). In seven of theses samples more than one serotype/serogroup was detected. Thus, a total of 183 serotypes/serogroups were identified. Eleven additional isolates were found to be non-encapsulated, and hence non-typeable (Figure 1). The combined results of the 194 identified serotypes/serogroups (including non-typeable) from a total of 186 pneumococcal isolates are shown in Figure 3.

The most prevalent serogroup was 6, present in 22 % (41/186) of the samples. Among the serogroup 6 isolates, 18 were further identified as serotype 6B and nine as serotype 6A, whereas the remaining 14 samples could not be subjected to further analysis due to low DNA concentration. The second most common serotype/serogroup was 15B/C which was present in 11 % (21/186) of the isolates followed by serotype 19F (11 %, 20/186), serotype 23F (8 %, 15/186) and serotype 19B (7 %, 13/186) (Figure 3).

The proportion of serotypes/serogroups included in PCV13 decreased from 56 % (40/71) in 2013 to 23 % (13/56) in 2015 (CI (95%) 0.056-0.346, not adjusted for multiplicity). Thus, in 2015 the

serotypes/serogroups not included in PCV13, including non-typeable isolates, were more prevalent (77 %) than the vaccine types (23 %). Moreover, whilst only one non-typeable isolate was found in 2013 and 2014 respectively, nine non-typeable pneumococci were identified in 2015 (Figure 3).

Among isolates with serotypes/serogroups included in the PCV13 penicillin non-susceptibility was equally common as penicillin susceptibility (50% vs. 50%, 36/78 for both), whilst the prevalence of penicillin non-susceptibility among non-PCV13 serotypes/groups (including non-typeable) was 39 % (38/98) (Figure 4). Most serotype/serogroup 15B/C isolates were susceptible to penicillin; on the contrary, almost all serotype 19B isolates had reduced susceptibility against penicillin (92 %, 12/13) (Figure 4). The proportion of penicillin non-susceptible pneumococci increased significantly among non-PCV13 serotypes/serogroups from 19 % (6/31) in 2013 to 50 % (20/40) in 2015 (CI (95 %) 0.098-0.515, not adjusted for multiplicity) (Additional file 4).

Discussion

This study has explored carriage of *S. pneumoniae* in children under two years of age residing in Moshi, Tanzania during a three-year period directly after introduction of the PCV13. Studies on the carriage rates of *S. pneumoniae* in healthy subjects are important in order to understand the dynamics of the population with respect to antibiotic resistance and serotype distribution. In our study, the pneumococcal carriage detected by culture was 31 %, which is in accordance with a previous study in Dar es Salaam, Tanzania, reporting a 35 % pre-vaccination pneumococcal carriage rate (Moyo, Steinbakk et al. 2012), and in Ghana, where the pneumococcal carriage among children was 34 % (Dayie, Arhin et al. 2013). However, similar studies in other regions of Africa have reported higher pneumococcal carriage both pre- and post-vaccination (Hammitt, Akech et al. 2014, Kobayashi, Conklin et al. 2017; Mills, Twum-Danso et al. 2015; Dube, Ramjith et al. 2018; Adetifa, Antonio et al. 2012). This variation can be attributed to differences in the study populations and consequently more or less exposure to risk factors as well variances in the detection methods (Bogaert, De Groot et

al. 2004, Satzke, Turner et al. 2013). For example, we did not use STGG medium for nasopharyngeal swab transport as recommended (Satzke, Turner et al. 2013), which may have led to the loss of viable pneumococci between sampling and culture. However, such loss would most likely be randomly spread between antibiotic susceptible and non-susceptible strains and should thus not affect the main results of this study.

The higher rate of pneumococcal colonization in children with signs of respiratory tract infection corroborates previous epidemiological studies (Abdullahi, Karani et al. 2012; Mills, Twum-Danso et al. 2015), as well as experimental studies performed in mice models that showed an increased adhesion of *S. pneumoniae* to epithelial cells after viral infection (Nita-Lazar, Banerjee et al. 2015; Smith, Sandrini et al. 2014). Our results also confirm an increase of pneumococcal carriage before the age of 2 years as previously reported (Abdullahi, Karani et al. 2012; Bogaert, De Groot et al. 2004). Among risk factors associated with pneumococcal carriage, exposure to household air pollution has shown to be relevant (Gordon, Bruce et al. 2014). Households included in our study would typically rely on several different fuels, stoves and locations for cooking which may vary according to changing needs or seasonal variations. Exposure to household air pollution induce inflammation of the respiratory tract and has been associated with pneumococcal colonization in epidemiological studies (Gordon, Bruce et al. 2014; Rylance, Kankwatira et al. 2016) as well as in experimental studies *in vitro* and in mice (Hussey, Purves et al. 2017). In our study there was no significant association between pneumococcal carriage and use of solid fuels, however, very few households relied on clean fuels only. Investigating possible effects of household air pollution on colonizing microbes *in vivo* is complex. We thus suggest future studies to include individual measurements of smoke exposure (Gordon, Bruce et al. 2014).

Knowledge of antimicrobial use and susceptibility in human pathogens in Eastern Africa is limited. In our study, we report a significant increase of penicillin non-susceptible pneumococci carried by

children from 31 % in 2013 to 53 % in 2015. No isolates were found to be fully penicillin-resistant but 41% of the isolates showed reduced susceptibility to penicillin. This percentage is lower compared to pre-PCV13 studies in Tanzania that reported 69 % and 68% of penicillin non-susceptibility in colonizing pneumococci in Dar es Salaam and Moshi, respectively (Moyo, Steinbakk et al. 2012; Bles, de Mast et al. 2015). However, this is in line with the situation in Ghana after introduction of the PCV13 (Dayie, Arhin et al. 2013). It is possible that penicillin non-susceptibility in healthy children in northern Tanzania was higher prior to introduction of the PCV13, as indicated by previous studies, and decreased as a result of the vaccine introduction. However, the number of isolates in Dar es Salaam (Moyo, Steinbakk et al. 2012) and Moshi (Bles, de Mast et al. 2015) was small and in Moshi the sampled children were born to HIV-positive mothers, this group possibly being more exposed to antibiotics due to increased sensitivity to infection. In addition, our sampling started shortly after the PCV13 introduction, and a dramatic drop in non-susceptibility of carried pneumococci is less likely to occur soon after the vaccine implementation.

Increased number of siblings was found to be an independent risk factor for carriage of pneumococci with reduced susceptibility to penicillin, this being closely related to previously reported factors such as crowding and day-care attendance (Kristinsson 1997). Girls were also more commonly colonized with penicillin non-susceptible pneumococci as shown in the multivariable model. As far as we know this has only been shown in one previous study conducted in Israel (Yagupsky, Porat et al. 1998), whilst several other studies show no association (Katsarolis, Poulakou et al. 2009; Stacevičienė, Petraitienė et al. 2016; Samore, Magill et al. 2001; Moyo, Steinbakk et al. 2012).

Despite a possible increase in intermediate penicillin resistance, and depending on the site of infection, penicillin can still be used for the treatment of pneumococcal infections in Tanzania (EUCAST 2018).

However, for more severe infections such as meningitis, ampicillin/gentamicin or ceftriaxone have been recommended as first-line treatment (WHO 2013).

In our study, non-susceptibility to erythromycin was higher (15 %) compared to studies in Dar es Salaam and in Moshi in 2010 that reported 6.0 % and 3.8 % of non-susceptibility, respectively (Moyo, Steinbakk et al. 2012; Bles, de Mast et al. 2015). This results implies an increase of almost fourthfold for pneumococci non-susceptible to erythromycin in Moshi in the last years. Macrolides, such as erythromycin, are commonly used in adults and children to treat acute lower respiratory tract infections since it is also effective against atypical agents such as *Mycoplasma pneumoniae* and *Legionella spp* (Ministry of Health and Social Welfare 2013). In our study, erythromycin was the third most commonly used antibiotic in the children after amoxicillin and trimethoprim-sulfamethoxazole. In addition, we also show high pneumococcal resistance to trimethoprim-sulfamethoxazole (97 %), which is similar to several studies from the region (Bles, de Mast et al. 2015; Kobayashi, Conklin et al. 2017; Moyo, Steinbakk et al. 2012). This antibiotic was previously considered as a first-line treatment for pneumonia, and usage of this antibiotic is still common for treatment of respiratory infections, probably due to persisting treatment traditions. Moreover, trimethoprim-sulfamethoxazole is widely used as a prophylactic treatment in HIV-exposed infants and HIV-positive individuals in resource-limited settings (WHO 2014a).

High use of prescribed antibiotics was observed during the study. A large majority of children (87 %) treated during the week prior to sampling were receiving the antibiotic through prescription by a clinician. However, only one third of the children in whom antibiotics were prescribed for respiratory tract symptoms presented fast or laboured breathing, i.e. signs of pneumonia which indicates need for antibiotics, according to their parent or guardian. These results confirm previous findings of inappropriate prescribing practices by physicians in Moshi (Gwimile, Shekalaghe et al. 2012) which

could be affected by perceived patient demand and be linked to cultural habits (Radyowijati and Haak 2003).

All information on antibiotic prescription and use was collected from the parent/guardian and complimented by reviewing the child's medical log when possible. Effort was put on avoiding confusion with other medicines by asking for the name of the antibiotic. Rates of antibiotic use based solely on parental recall is often underestimated, as shown by previous studies comparing parents' reported use of antibiotics with detected antibiotics in the urine of the child (Driscoll, Bhat et al. 2012; Khennavong, Davone et al. 2011; Sombrero, Sunico et al. 1999). Moreover, high use and miss-use of antibiotics in the community is one of the drivers of increased antibiotic resistance. Thus, although there is uncertainty in the reported use of antibiotics, there are reasons to believe a more rational use of antibiotics in children in northern Tanzania is of major public health importance.

In our study, serotype/group 6, 19F, 23F and 14 were the most prevalent serotypes/serogroups of those included in PCV13. These serotypes are considered colonisers and tend to be carried within the nasopharynx for prolonged periods of time compared with more invasive and immunogenic serotypes such as serotypes 1 and 3 (Dube, Ramjith et al. 2018; Sleeman, Griffiths et al. 2006). Among the non-PCV13 serotypes/groups 15B/C, 19B and 10A were the most prevalent. Serotype/group 15B/C has been reported as one of the predominant serotypes/groups recovered after PCV13 in both low and high-income countries (Devine, Cleary et al. 2017; Ho, Chiu et al. 2015; Dube, Ramjith et al. 2018). Other studies in Africa have reported a high prevalence of serotype/group 15B/C, 10A, 11 and 19A (Birindwa, Emgard et al, 2018; Kwambana-Adams, Hanson et al. 2017; Dube, Ramjith et al. 2018). However, the relative high prevalence of serotype 19B in this study was unexpected and has to our knowledge not been reported in other recently published African studies.

Apart from protecting vulnerable subjects from invasive pneumococcal disease, the PCV13 was also designed to target some serotypes more commonly associated with high non-susceptibility (Kyaw, Lynfield et al. 2006). Following introduction of PCV, a change from PVC-serotypes to non-PCV serotypes in the colonization of the nasopharynges has been shown (Croucher, Finkelstein et al. 2013; Hammitt, Akech et al. 2014), which was also demonstrated in this study. In the PCV era, colonizing pneumococci is thus exposed to selective pressure from both the vaccine and administered antibiotics. This may consequently select for those resistant strains not included in the vaccine (Danino, Givon-Lavi et al. 2018; Croucher, Chewapreecha et al. 2014; Keenan, Klugman et al. 2015). In line with this, our study shows a notable increase of pneumococci with reduced susceptibility to penicillin among non-PCV13 serotypes in 2015 (50 %) compared to 2013 (19 %). This highlights the need for continued surveillance of serotype distribution in colonization and disease, in association with antibiotic susceptibility, also after vaccine implementation. The second most common non-PCV13 serotype was unexpectedly 19B, of which almost all were non-susceptible to penicillin (12 out of 13). Unfortunately, further analysis of possible clonality among these isolates could not be performed due to scarce supply of genetic material.

Conclusions

Penicillin non-susceptibility increased in colonizing *S. pneumoniae* in northern Tanzania during a three-year period soon after introduction of the PCV13 vaccine. However, non-susceptibility to amoxicillin/ampicillin and ceftriaxone was still low. Measures to ensure rational use of antibiotics and more effective systems for surveillance of antibiotic resistance and serotype distribution are needed to assure continued effectual treatment of pneumococcal disease.

Declarations

Ethics approval and consent to participate

The study was approved by the Kilimanjaro Christian Medical University College Research Ethics and Review Committee in Moshi, Tanzania (No. 661 and 809), the National Institute for Medical Research

in Dar es Salaam (Vol. IX/2363) and the Regional Ethics Committee in Gothenburg (413-15). The study was carried out in accordance with existing ethical guidelines in Tanzania and Sweden. The Municipal Medical Doctor of Health at Moshi Municipal Council was informed of the study and gave permission to visit the health facilities. Informed oral and written consent was obtained from the accompanying parent or guardian of each child included in the study.

Authors' contributions

SS, SEM, BN and RA designed and sought ethical permission for the study. ME, JB, SF and FJ together with native research nurses obtained consent from the parents or guardians to participate, acquired information for the questionnaires and collected the samples. Further, ME, JB, SF and FJ performed the laboratory work together with local laboratory technicians at KCMC, Moshi, Tanzania. LGS, RN, SG, VM and ME performed the biomolecular analyses at Gothenburg University, Gothenburg, Sweden. ME and LGS analysed the data with close communication with SS and RA. ME was mainly responsible for writing the manuscript which was critically revised by SS, RA, SEM, NB and DM. All authors read and approved the final manuscript.

Consent for publication

Not applicable.

Availability of data and material

The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

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scholarship. The funding body had no role in the design of the study, the collection, analysis, interpretation of data nor in the writing of the manuscript.

Competing interest

The authors declare they have no competing interests.

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Figures

Figure 1. Schematic representation of the analyses performed in Moshi, Tanzania and in Gothenburg, Sweden, respectively, and the number of isolates included in each analysis.

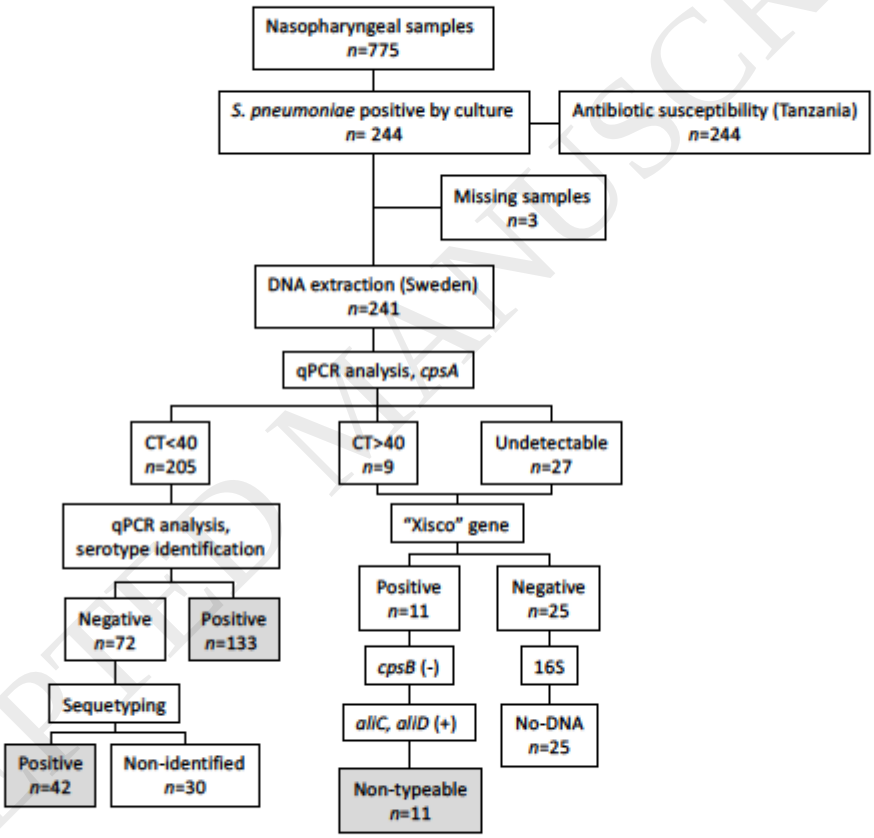


Figure 2. All pneumococcal isolates (*n*=244) from 775 children were tested for antibiotic non-susceptibility in Tanzania. Non-susceptibility to penicillin G (MIC >0.06 mg/L), ampicillin and ceftriaxone were determined by E-tests. Penicillin non-susceptibility increased significantly between 2013-2014 *(*p*=0.042) and 2013-2015 **(*p*=0.033). [a]Trimethoprim-Sulfamethoxazole. [b]Used for screening of fluoroquinolone resistance i.e. levofloxacin and moxifloxacin. [c]Multi-drug resistant, non-susceptible against ≥3 classes of antibiotics.

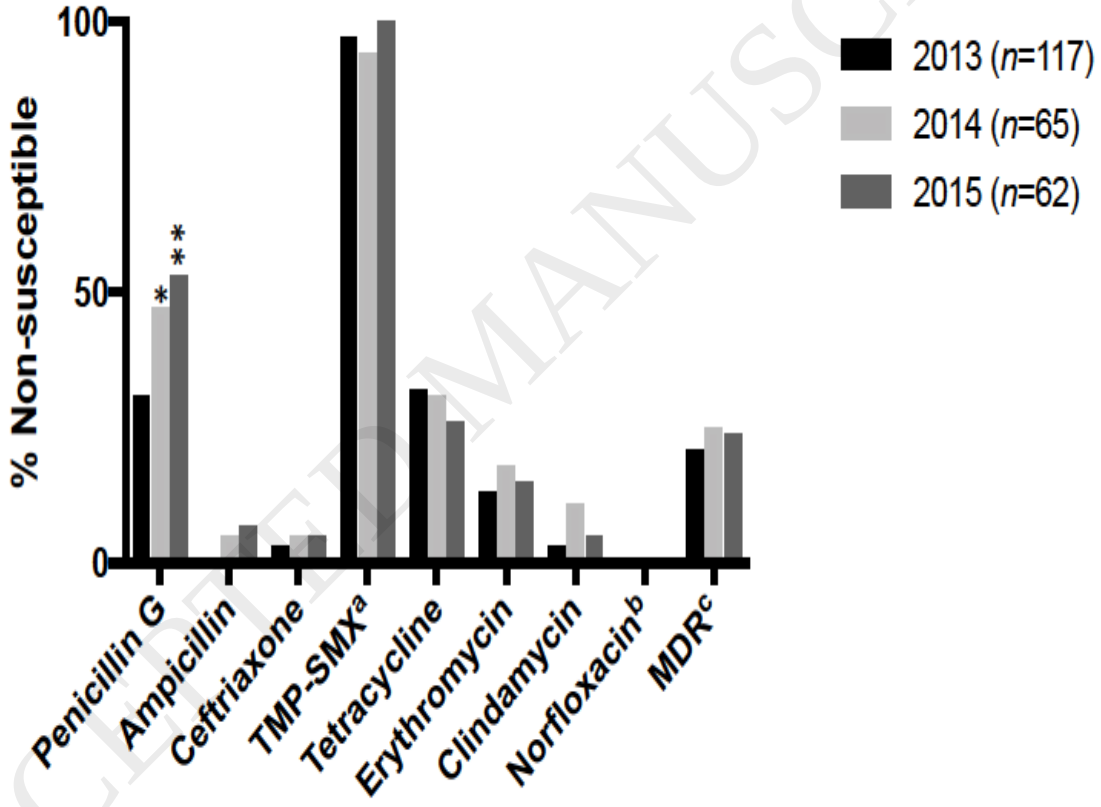


Figure 3. A total of 194 serotypes/serogroups, including 11 non-typeable, were identified by molecular methods performed in Sweden in 186 pneumococcal isolates obtained from children below 2 years of age in Tanzania during 2013-2015.

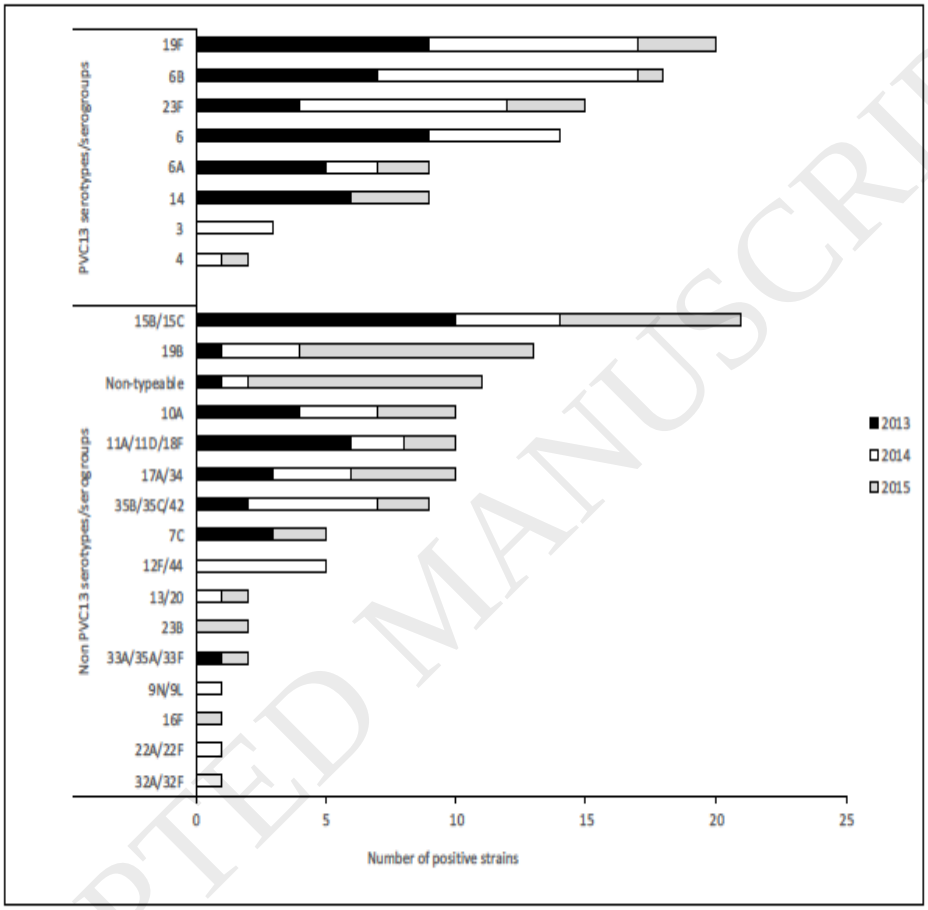
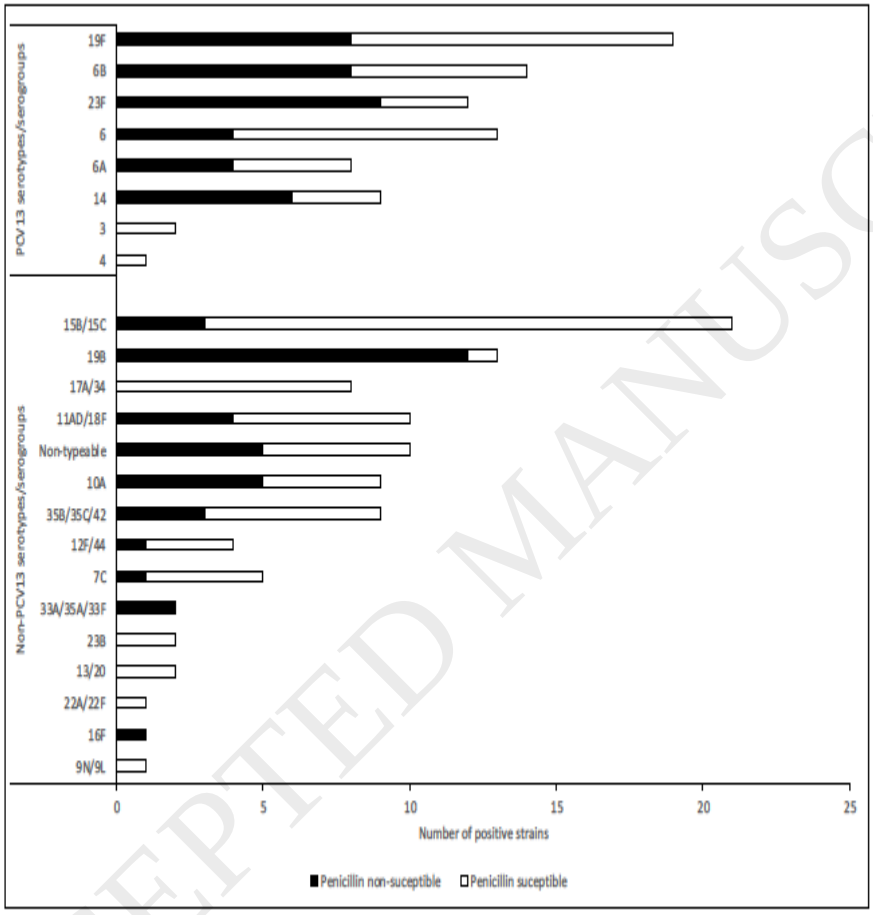


Figure 4. Distribution of penicillin-susceptible and penicillin non-susceptible pneumococcal isolates among PVC13 and non-PVC13 serotypes (*n*=176). A total of 186 isolates were serotyped/grouped; in seven isolates more than one serotype/group was identified and in three isolates, determination of penicillin susceptibility was incomplete; these isolates (*n*=10) were excluded in the analysis.



Tables

Table 1. Characteristics of the recruited children (*n*=775), as reported by the parent/guardian

	2013 (*n*=338)	2014 (*n*=224)	2015 (*n*=213)	Total (*n*=775)
Characteristic	*n* (%)	*n* (%)	*n* (%)	*n* (%)
Residence				
Bondeni	51 (15)	0 (0)	35 (16)	86 (11.1)
Njoro	95 (28)	0 (0)	46 (22)	141 (18.2)
Pasua	192 (57)	0 (0)	132 (62)	324 (41.8)
Majengo	0 (0)	105 (47)	0 (0)	105 (13.5)
Rau	0 (0)	88 (39)	0 (0)	88 (11.4)
Shirimatunda	0 (0)	31 (14)	0 (0)	31 (4.0)
Age				
<6 months	132 (39)	91 (41)	50 (23)	273 (35.2)
6-11 months	103 (30)	85 (38)	92 (43)	280 (36.1)
12-17 months	69 (20)	35 (16)	51 (24)	155 (20.0)
18-23 months	34 (10)	13 (5.8)	20 (9.4)	67 (8.6)
Sex				
Girl	163 (48)	109 (49)	102 (48)	374 (48.3)
Boy	175 (52)	115 (51)	111 (52)	401 (51.7)
Reason for attending at health facility				
Vaccination	101 (30)	49 (22)	23 (11)	173 (22.3)
Growth monitoring	135 (40)	135 (60)	119 (56)	389 (50.2)
OPD clinic (child is sick)	76 (22)	39 (17)	61 (29)	176 (22.7)
Other	26 (7.7)	1 (0.5)	10 (4.7)	37 (4.8)
Nutritional status				
Underweight[a]	12 (3.6)	8 (3.6)	11 (5.2)	31 (4.0)
Stunted[b]	110 (33)	83 (37)	43 (20)	236 (30.5)
Breastfeeding, children <6 months (*n*=273)				
Exclusive	34 (26)	57 (63)	26 (52)	117 (42.9)
Partly breastfed	95 (72)	34 (37)	24 (48)	153 (56.0)
Not breastfed	3 (2.3)	0 (0)	0 (0)	3 (1.1)
Breastfeeding, children 6-23 months (*n*=502)				
Exclusive or partly	184 (89)	119 (89)	146 (90)	449 (89.4)
Not breastfed	22 (11)	14 (11)	17 (10)	53 (10.6)
Presenting with symptoms of RTI[c]	185 (55)	110 (49)	116 (54)	411 (53.0)
With fever	47 (25)	24 (22)	38 (33)	109 (26.5)
Without fever	138 (75)	86 (78)	78 (67)	302 (73.5)

Presumed pneumonia[d], last 3 months	22 (6.5)	29 (13)	39 (18)	90 (11.6)
Current or previous diseases				
Malaria (confirmed)	90 (27)	14 (6.3)	38 (18)	142 (18.3)
Diarrhoea	147 (43)	26 (12)	18 (8.5)	191 (24.6)
Level of education, mother				
Primary education and below	230 (68)	128 (57)	135 (63)	493 (63.6)
Secondary school	102 (30)	70 (31)	70 (33)	242 (31.2)
University	4 (1.2)	14 (6.3)	4 (1.9)	22 (2.8)
Occupational/other	2 (0.6)	12 (5.4)	4 (1.9)	18 (2.3)
Level of education, father				
Primary education and below	187 (55)	110 (49)	112 (53)	409 (52.8)
Secondary school	123 (36)	69 (31)	74 (35)	266 (34.3)
University	22 (6.5)	29 (13)	9 (4.2)	60 (7.7)
Occupational/other	5 (1.5)	15 (6.7)	7 (3.3)	27 (3.5)
Unknown	1 (0.3)	1 (0.4)	11 (5.2)	13 (1.7)
Crowding				
≥3 siblings	49 (14)	32 (14)	26 (12)	107 (13.8)
≥3 people/room in household	140 (41)	72 (32)	86 (40)	298 (38.5)
≥3 people sharing bedroom with the child	69 (20)	37 (17)	82 (38)	188 (24.3)
≥8 people/household	11 (3.3)	16 (7.1)	6 (2.8)	33 (4.3)
Solid fuel used for cooking[e]	274 (81)	191 (85)	193 (91)	658 (84.9)
Adult smoking in the household	49 (14)	38 (17)	27 (13)	114 (14.7)
Child vaccinated with PCV13				
<12 months				
Yes, fully	140 (60)	105 (60)	122 (86)	367 (66)
Yes, partial	65 (28)	41 (23)	17 (12)	123 (22)
No	30 (13)	30 (17)	3 (2.1)	63 (11)
12-23 months				
Yes, fully	3 (2.9)	44 (92)	71 (100)	118 (53)
Yes, partial	0 (0)	0 (0)	0 (0)	0 (0)
No	100 (97)	4 (8.3)	0 (0)	104 (47)
Previous antibiotic use in the child				
≤1 week	49 (14)	49 (22)	52 (24)	150 (19.4)
Prescribed	*35 (71)*	*46 (94)*	*50 (96)*	*131 (87.3)*
Over the counter	*14 (29)*	*3 (6.1)*	*2 (3.8)*	*19 (12.7)*
>1-4 weeks	82 (24)	44 (20)	62 (29)	188 (24.3)
>4-12 weeks	82 (24)	54 (24)	79 (37)	215 (27.7)
Reason for antibiotic use (≤1 week, *n*=101)				
RTI with fast/difficult breathing[d]		9 (18)	17 (33)	26 (25.7)
RTI without fast/difficult breathing		25 (51)	26 (50)	51 (50.5)

Other		15 (31)	9 (17)	24 (23.8)

[a]Weight-for-age <-2SD. Total *n*=770 (2013 *n*=336, 2014 *n*=223, 2015 *n*=211).

[b]Length-for-age <-2SD. Total *n*=767 (2013 *n*=335, 2014 *n*=220, 2015 *n*=212).

[c]Respiratory tract infection (cough, runny nose or fast/difficult breathing with or without fever)

[d]Respiratory tract infection with fast/difficult breathing (according to parent/guardian)

[e]Firewood or charcoal

Table 2. Carriage of *Streptococcus pneumoniae* in relation to risk factors

(Total no. of isolates: 244, total no. children: 775, overall pneumococcal carriage rate: 31 %)

		Univariable analysis			Multivariable analysis[a]		
	No. carriers/ Total no. (%)	OR	CI (95%)	*p*-Value	OR	CI (95%)	*p*-Value
Age (closer to 2 years)[b]		1.04	1.01-1.07	0.004	1.04	1.01-1.07	0.008
Sex, girl	128/374 (34)	1.28	0.94-1.73	*NS*	1.28	0.94-1.76	*NS*
Current symptoms of RTI[c]	146/411 (36)	1.50	1.10-2.03	0.010	1.50	1.09-2.08	0.014
Presumed pneumonia[d], last 3 months	25/90 (28)	0.82	0.50-1.33	*NS*	0.86	0.51-1.44	*NS*
History of gastrointestinal disease	68/191 (36)	1.28	0.91-1.81	*NS*	0.93	0.62-1.40	*NS*
Underweight[e]	10/31 (32)	1.03	0.48-2.22	*NS*	0.97	0.43-2.19	*NS*
Stunted[f]	79/236 (33)	1.13	0.81-1.56	*NS*	1.08	0.76-1.54	*NS*
Antibiotic use in the child, last 7 days	40/150 (27)	0.75	0.50-1.12	*NS*	0.67	0.44-1.03	*NS*
Mother's education, ≤7 years	168/493 (34)	1.40	1.02-1.93	0.040	1.05	0.72-1.54	*NS*
Father's education, ≤7 years	142/411 (35)	1.37	1.01-1.87	0.044	1.18	0.83-1.68	*NS*
Adult smoking in the household	38/114 (33)	1.10	0.72-1.69	*NS*	0.96	0.62-1.49	*NS*
Solid fuel[g] used for cooking	216/658 (33)	1.55	0.99-2.45	*NS*	1.39	0.86-2.24	*NS*

Number of siblings[b]		1.14	1.01-1.29	0.031	1.11	0.97-1.27	*NS*
Crowding[b, h]		1.18	1.03-1.34	0.015	1.13	0.99-1.30	*NS*

[a]Adjusted for year of sampling and covariates included in the univariable analysis

[b]Continuous variables (all other variables are categorical)

[c]Respiratory tract infection (cough, runny nose, fast or laboured breathing with or without fever)

[d]Respiratory tract infection with fast or laboured breathing (according to parent/guardian)

[e]Weight-for-age <-2SD

[f]Length-for-age <-2SD

[g]Firewood or charcoal

[h]People per room in household

Table 3. Carriage of penicillin non-susceptible *Streptococcus pneumoniae* (PNSP) in relation to risk factors

(Total no. of PNSP: 98, total no. children: 775, overall carriage of PNSP: 13 %)

		Univariate analysis			Multivariate analysis[a]		
	No. carriers/ Total no. (%)	OR	CI (95%)	*p*-Value	OR	CI (95%)	*p*-Value
Age (closer to 2 years)[b]		1.01	0.97-1.05	*NS*	1.02	0.98-1.07	*NS*
Sex, girl	55/374 (15)	1.44	0.94-2.20	*NS*	1.49	0.96-2.33	0.045
Current symptoms of RTI[c]	55/411 (13)	1.15	0.75-1.77	*NS*	1.20	0.77-1.87	*NS*
Presumed pneumonia[d], last 3 months	11/90 (12)	0.96	0.49-1.87	*NS*	0.94	0.45-1.91	*NS*
History of gastrointestinal disease	25/191 (12)	1.05	0.65-1.72	*NS*	1.23	0.70-2.16	*NS*
Underweight[e]	3/31 (10)	0.73	0.22-2.44	*NS*	0.74	0.21-2.61	*NS*
Stunted[f]	29/236 (12)	0.94	0.59-1.49	*NS*	0.97	0.59-1.60	*NS*
Antibiotic use in the child, last 7 days	16/150 (11)	0.79	0.45-1.40	*NS*	0.66	0.36-1.21	*NS*
Mother's education, ≤7 years	68/493 (14)	1.34	0.85-2.12	*NS*	1.10	0.64-1.86	*NS*
Father's education, ≤7 years	56/411 (14)	1.27	0.82-1.97	*NS*	1.15	0.70-1.89	*NS*
Adult smoking in the household	11/114 (10)	0.71	0.36-1.37	*NS*	0.60	0.30-1.18	*NS*

Solid fuel[g] used for cooking	87/658 (13)	1.47	0.76-2.84	*NS*	1.18	0.59-2.35	*NS*
Number of siblings[b]		1.24	1.06-1.46	0.008	1.23	1.03-1.46	0.019
Crowding[b, h]		1.12	0.94-1.34	*NS*	1.09	0.90-1.32	*NS*
Fully vaccinated with PCV13	69/485 (14)	1.97	1.06-3.66	0.033	2.04	0.99-4.16	*NS*

[a]Adjusted for year of sampling and covariates included in the univariable analysis

[b]Continuous variable (all other variables are categorical)

[c]Respiratory tract infection (cough, runny nose, fast or laboured breathing with or without fever)

[d]Respiratory tract infection with fast or laboured breathing (according to parent/guardian)

[e]Weight-for-age <-2SD

[f]Length-for-age <-2SD

[g]Firewood or charcoal

[h]People per room in household

Table 4. Total antibiotic susceptibility of the pneumococcal isolates (*n*=244[a])

Antimicrobial agent	Intermediate, *n*	Resistant, *n*	Non-susceptible[b], *n* (%)
Penicillin V	0	112	112 (46)
Penicillin G	98	0	98 (41)
Ampicillin	8	0	8 (3)
Ceftriaxone	9	0	9 (4)
TMP-SMX[c]	11	225	236 (97)
Tetracycline	20	54	74 (30)
Erythromycin	15	21	36 (15)
Clindamycin	-	14	14 (6)
Norfloxacin	-	0	0 (0)

[a]Penicillin G, ampicillin, ceftriaxone *n*=240, erythromycin *n*=243, clindamycin *n*=241

[b]Intermediate or resistant

[c]Trimethoprim-sulfamethoxazole