International Journal of Infectious Diseases 81 (2019) 156–166

Contents lists available at ScienceDirect

International Journal of Infectious Diseases

journal homepage: www.elsevier.com/locate/ijid

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

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ARTICLE INFO

Article history:
Received 29 July 2018
Received in revised form 18 January 2019
Accepted 19 January 2019
Corresponding Editor: Eskild Petersen, Aarhus, Denmark

Keywords:
Streptococcus pneumoniae
Drug resistance
Bacterial
Air pollution
Pneumococcal vaccines
Nasopharyngeal colonization

ABSTRACT

Objectives: To determine the antibiotic susceptibility and serotype distribution of colonizing *Streptococcus pneumoniae* in Tanzanian children. Serial cross-sectional surveys were performed following the national introduction of the 13-valent pneumococcal conjugate vaccine (PCV13) in December 2012.

Methods: A total of 775 children less than 2 years of age were recruited at primary health centres in Moshi, Tanzania between 2013 and 2015, and samples were obtained from the nasopharynx. *S. pneumoniae* were isolated by culture and tested for antibiotic susceptibility by disc diffusion and E-test methods; 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 were 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 introduction of PCV13. Measures to ensure rational use of antibiotics and more effective systems for surveillance of antibiotic resistance and serotype distribution are needed to assure continued effective treatment of pneumococcal disease.

Introduction

In the era of pneumococcal conjugate vaccines (PCVs), *Streptococcus pneumoniae*, or the pneumococcus, remains an important cause of bacterial pneumonia, sepsis, and meningitis in children (Wahl et al., 2018). Nasopharyngeal colonization with pneumococci is most common in preschool children; it precedes infection and enables horizontal spread of the bacteria (Bogaert 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 et al., 2012).

High antibiotic use is associated with an increased risk of nasopharyngeal carriage of penicillin-non-susceptible pneumococci (PNSP) in children (Arason et al., 1996; Kobayashi et al., 2017; Melander et al., 1998). According to the World Health Organization (WHO) Integrated Management of Childhood Illness, oral amoxicillin is the drug of choice for the treatment of childhood

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https://doi.org/10.1016/j.ijid.2019.01.035
1201-9712/

pneumonia at the primary health care level (WHO, 2014b). Children aged 2 months to 5 years presenting with fast breathing or chest indrawing, who do not improve after treatment with a bronchodilator in the case of wheezing, are assumed to be suffering 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 et al., 2012).

The PCV was developed to generate an appropriate immune response in small children and so defend against the seven (PCV7), 10 (PCV10), or 13 (PCV13) most disease-causing pneumococcal serotypes. In December 2012, the 13-valent PCV (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). PCV13 has been shown to dramatically reduce the incidence in children of invasive pneumococcal disease caused by the included serotypes (von Gottberg et al., 2014; Cutts et al., 2005) and to lower the incidence of radiologically proven pneumonia in South Africa and the Gambia (Klugman et al., 2003; Cutts et al., 2005; Mackenzie et al., 2017). Soon after the introduction of PCV10 in Kilifi, Kenya, carriage of vaccine serotypes in children less than 5 years of age decreased by 64%, also proving herd effect in older children and adults (Hammitt et al., 2014). The proportional impact of the vaccine depends on the 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 et al., 2012).

Both PCV7 and PCV13 have been shown to reduce antibiotic-resistant invasive pneumococcal disease in countries such as South Africa, the USA, and Canada (Klugman et al., 2003; Tomczyk et al., 2016; Tyrrell et al., 2009). However, the effect of PCV13 on antibiotic non-susceptibility in colonizing and/or invasive pneumococci is less known in large parts of Sub-Saharan Africa, due to the lack of routine culture and antibiotic susceptibility testing (Balsells et al., 2017; Hackel et al., 2013; Williams 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, 2017GBD, 2017GBD 2015 LRI Collaborators, 2017; Gordon et al., 2014). Exposure to toxic pollutants, such as particulate matter, has been shown to affect the defence against microorganisms at all levels of the respiratory tract (Barregard et al., 2008; Gordon et al., 2014; Hawley and Volckens, 2013; Rylance 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 et al., 2017; Rylance et al., 2016).

The aim of this study was to determine the antibiotic susceptibility and serotype/serogroup distribution of colonizing pneumococci in Tanzanian children during a 3-year period shortly after the introduction of PCV13 in Moshi, Tanzania. A further aim was to assess the epidemiology of pneumococcal carriage, including its associations with household air pollution and antibiotic use.

Methods

Recruitment of children

Serial cross-sectional surveys were performed in October and November 2013, February and March 2014, and February to 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. Samples were collected at six different health facilities, chosen to represent different geographical locations within the wider district. These included dispensaries (Bondeni, Njoro, Rau) and health centres (Pasua, Majengo, Shirimatunda) (**Supplementary material**, Fig. S1). All children less than 2 years of age attending the study health facilities with their parents or guardians for medical attention, including routine growth monitoring, were invited to participate in the study. The parents or guardians responded to a questionnaire for the collection of socio-demographic information and the child's health status. 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 at the household level, parents or guardians participating in 2015 were asked additional questions to obtain information on the use of stoves, cooking location, and construction of the household building. The current weight and length of the child were recorded. Due to greater numbers of patients attending health centres in areas with a 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 following the standard procedure (Satzke et al., 2013), using a Blue-cap E-swab (Copan Diagnostics Inc., Murrieta, USA). The samples were stored in a cool box and were transported to the Clinical Laboratory at Kilimanjaro Christian Medical Centre (KCMC), Moshi, Tanzania, for culture, all within 6 h.

Isolation and identification of S. pneumoniae

At KCMC laboratory, the samples were inoculated on goat blood agar (HiMedia, Mumbai, India) and incubated at 37 °C in sealed containers (Oxoid Ltd., Hampshire, UK) along with a CO_2 gas pack (BD GasPak EZ CO_2 Container System) and a CO_2 indicator (BD CO_2 Indicator 0.5 ml) for 16–20 h, and a further 40–44 h 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 KCMC laboratory and was determined by disc diffusion and E-test methods according to the procedures and breakpoints published by the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2018). The tests were performed on Mueller–Hinton agar (Oxoid), supplemented with 5% added goat blood and 20 mg/l β-NAD (beta-Nicotinamide adenine dinucleotide, Applichem, Darmstadt, Germany). Inoculated plates were incubated at 37 °C in a CO_2 environment, as described above. The following antimicrobial discs were used: oxacillin (screening disc for β-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 disc clearance zone was less than 20 mm, the minimum inhibitory concentration (MIC) was determined by E-test for penicillin G, ampicillin, and ceftriaxone (each at 0.016–256 μg/ml; BioMérieux, Marcy l'Etoile, France). Resistant and intermediate isolates were all referred to as non-susceptible. Multi-drug resistance was defined as non-susceptibility to three or more classes of antimicrobial agents including the β-lactams (i.e., penicillin G, ampicillin, or ceftriaxone) (Finkelstein et al., 2003; Moyo 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 the 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.

The identification of *S. pneumoniae* was performed via the detection of the pneumococcal capsule coding gene *cpsA* by real-time quantitative PCR (qPCR) (preprint available at https://www.biorxiv.org/content/early/2018/09/12/415422). A cycle threshold (Ct) value of <40 was considered a positive result. Samples that were negative for the capsule gene were further tested for the presence of the 'Xisco' gene, shown to be a unique marker for the identification of *S. pneumoniae* (Salva-Serra 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 et al., 1997). A flow chart of the analyses performed is shown in Figure 1.

Serotyping of S. pneumoniae

Initially, the detection of 40 different serotypes was performed using a multiplex real-time PCR protocol published by the US Centers for Disease Control and Prevention (CDC) with slight modifications, as described previously (Birindwa 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 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 serotypes 6A and 6C, a 6-bp deletion in the *wzy* gene was detected. Differentiation of serotypes 6B and 6D was done by a single PCR analysis.

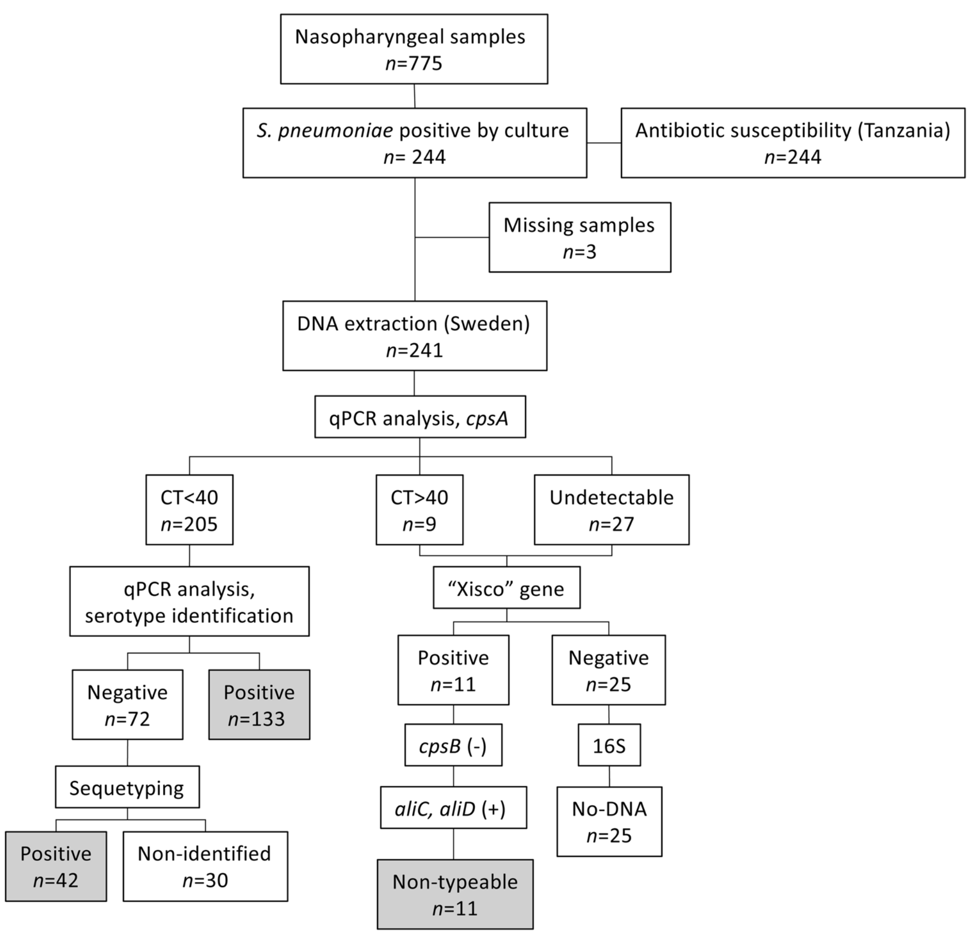

Figure 1. Schematic representation of the analyses performed in Moshi, Tanzania and in Gothenburg, Sweden, respectively, and the number of isolates included in each analysis.

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. (2012).

Statistical analysis

Univariable and multivariable logistic regression were performed to determine risk factors for pneumococcal carriage and for carriage of PNSP in all of the children included. 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 previous studies were considered covariates and were adjusted for in all multivariable models (Abdullahi et al., 2012; Arason et al., 1996; Bogaert et al., 2004; Kobayashi et al., 2017; Melander et al., 1998). The analyses were performed using IBM SPSS Statistics v. 25 (IBM Corp., Armonk, NY, USA) and the significance of coefficients was tested for using Wald's test; *p*-values of ≤0.05 were considered significant.

Results

Characteristics of the study population

Eight hundred parents or guardians were informed about the study and 23 of them refused to let their child participate. Two children were excluded because of the young age of the parent or guardian (<18 years). Thus a total of 775 children were included. The median age of the children was 8 months (range 0–24 months). Socio-economic 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 had been 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 3 months. Most antibiotics (87%, 131/150) consumed within 7 days prior to sampling had been 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 ($n = 51$) had presented with a cough and/or runny nose with or without fever according to the parent or guardian. Amoxicillin and ampicillin were the most commonly used antibiotics (**Supplementary material**, Fig. S2).

The majority (85%, $n = 658$) of households included during the period 2013–2015 used solid fuel for cooking, i.e. firewood or charcoal (Table 1). Most households included in 2015 (88%, 188/213) reported the use of more than one stove (**Supplementary material**, 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 (**Supplementary material**, Table S2). Most household buildings (75%, $n = 160$) were made of a material with high thermal mass such as adobe, baked bricks, or concrete (**Supplementary material**, Table S1).

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

Characteristic	2013 ($n = 338$) n (%)	2014 ($n = 224$) n (%)	2015 ($n = 213$) n (%)	Total ($n = 775$) 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				
Female	163 (48)	109 (49)	102 (48)	374 (48.3)
Male	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 per 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 per 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)

Table 1 (*Continued*)

Characteristic	2013 (n = 338) n (%)	2014 (n = 224) n (%)	2015 (n = 213) n (%)	Total (n = 775) n (%)
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)

OPD, outpatient department; RTI, respiratory tract infection; PCV, pneumococcal conjugate vaccine; SD, standard deviation.
[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.

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 the isolated pneumococci at KCMC laboratory in Tanzania. Both univariable and multivariable analyses showed that pneumococcal carriage increased significantly with age and during respiratory tract infection (Table 2). In the 2015 cohort, no association was found between the type of fuel used for cooking, construction of the roof, or material used to construct the walls and carriage of *S. pneumoniae* (**Supplementary material**, Table S3).

Antimicrobial resistance patterns of S. pneumoniae

The antimicrobial susceptibility pattern was determined at KCMC laboratory for the 244 pneumococcal isolates (Table 3). 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. None of the isolates were found to be resistant to penicillin G (MIC >2 mg/l), but 98 isolates were intermediate (MIC >0.06–2 mg/l), as determined using EUCAST breakpoints for pneumococcal infections other than meningitis (EUCAST, 2018). Thus, overall, 41% (98/240) of the isolates had MIC values greater than 0.06 mg/l for penicillin G and were therefore considered PNSP. A 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 53% (32/60) in 2015 (Figure 2). Multi-drug resistance (non-susceptibility to at least three or more classes of antimicrobial agents) was 23% overall (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 with pneumococci with reduced susceptibility to penicillin in the univariable and multivariable analyses (Table 4). Girls were more commonly colonized with PNSP, as shown in the multivariable analysis (Table 4).

Molecular identification of S. pneumoniae

Of the 244 isolates identified as *S. pneumoniae* by optochin susceptibility testing at 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-

Table 2
Carriage of *Streptococcus pneumoniae* in relation to risk factors (total number of isolates = 244; total number of children = 775; overall pneumococcal carriage rate = 31%).

	Carriers/total	Univariable analysis			Multivariable analysis[a]		
	n/n (%)	OR	95% CI	p-Value	OR	95% CI	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

OR, odds ratio; CI, confidence interval; NS, not significant; RTI, respiratory tract infection; SD, standard deviation.
[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 the household.

Table 3
Carriage of penicillin-non-susceptible *Streptococcus pneumoniae* (PNSP) in relation to risk factors (total number of PNSP = 98; total number of children = 775; overall carriage of PNSP = 13%).

	Carriers/total *n/n* (%)	Univariable analysis			Multivariable analysis[a]		
		OR	95% CI	*p*-Value	OR	95% CI	*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

OR, odds ratio; CI, confidence interval; NS, not significant; RTI, respiratory tract infection; PCV, pneumococcal conjugate vaccine; SD, standard deviation.
[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.

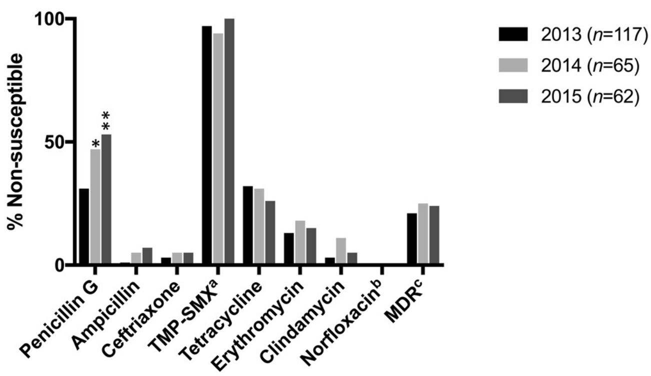

Figure 2. All pneumococcal isolates ($n = 244$) from 775 children were tested for antibiotic non-susceptibility in Tanzania. Non-susceptibility to penicillin G (MIC >0.06 mg/l), ampicillin, and ceftriaxone were determined by E-test. Penicillin non-susceptibility increased significantly between 2013 and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

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.

encapsulated or non-typeable *S. pneumoniae* by the presence of the 'Xisco' and *aliC/aliD* genes. For the remaining 25 samples, no PCR amplification could be achieved, possibly due to degradation of bacterial DNA during storage and transportation.

Serotype distribution

The serotypes or serogroups were determined for 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). More than one serotype/serogroup was detected in seven of these samples. 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.

Serogroup 6 was the most prevalent, 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; 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 (95% confidence interval 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 as common as penicillin susceptibility (50% vs. 50%, 36/78 for both), whilst the

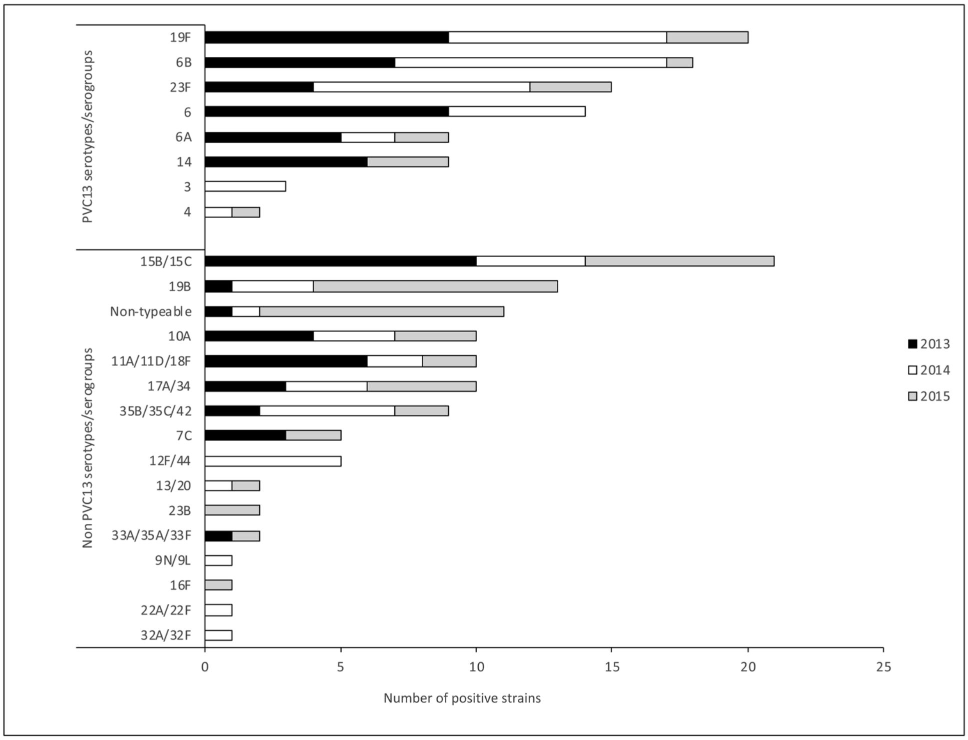

Figure 3. A total of 194 serotypes/serogroups, including 11 non-typeable, were identified by molecular methods performed in Sweden on 186 pneumococcal isolates obtained from children <2 years of age in Tanzania during 2013–2015.

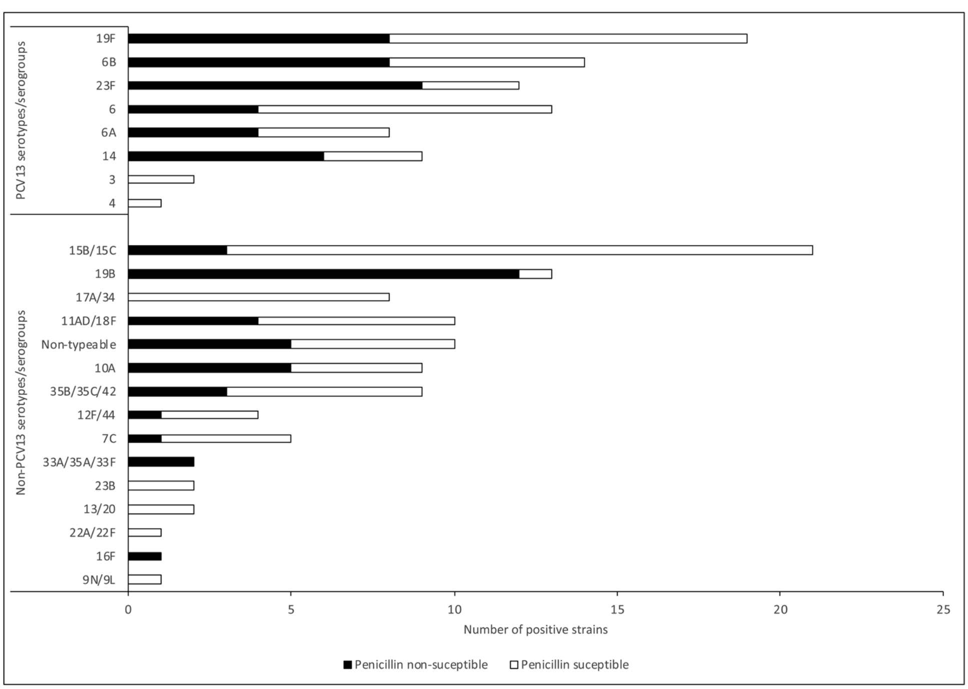

Figure 4. Distribution of penicillin-susceptible and penicillin-non-susceptible pneumococcal isolates among PVC13 and non-PVC13 serotypes ($n = 176$). A total of 186 isolates were serotyped/grouped. More than one serotype/group was identified in seven isolates, and determination of penicillin susceptibility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

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; in contrast, almost all serotype 19B isolates had reduced susceptibility to penicillin (92%, 12/13) (Figure 4). The proportion of PNSP increased significantly among non-PCV13 serotypes/serogroups from 19% (6/31) in 2013 to 50% (20/40) in 2015 (95% confidence interval 0.098–0.515, not adjusted for multiplicity) (**Supplementary material**, Fig. S3).

Discussion

This study explored carriage of *S. pneumoniae* in children under 2 years of age residing in Moshi, Tanzania during a 3-year period directly after the introduction of PCV13. Studies on 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 this study, the pneumococcal carriage detected by culture was 31%, which is in accordance with the results of a previous study performed in Dar es Salaam, Tanzania, which showed a 35% pre-vaccination pneumococcal carriage rate (Moyo et al., 2012), and a study performed in Ghana, where the pneumococcal carriage rate among children was 34% (Dayie et al., 2013). However, similar studies in other regions of Africa have reported higher pneumococcal carriage both pre- and post-vaccination (Hammitt et al., 2014; Kobayashi et al., 2017; Mills et al., 2015; Dube et al., 2018; Adetifa 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 used (Bogaert et al., 2004; Satzke et al., 2013). For example, STGG medium was not used for nasopharyngeal swab transportation, as recommended (Satzke 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 et al., 2012; Mills et al., 2015), as well as experimental studies performed in the mouse model that have shown an increased adhesion of *S. pneumoniae* to epithelial cells after viral infection (Nita-Lazar et al., 2015; Smith et al., 2014). The present study results also confirm an increase in pneumococcal carriage before the age of 2 years, as reported previously (Abdullahi et al., 2012; Bogaert et al., 2004). Among risk factors associated with pneumococcal carriage, exposure to household air pollution has been shown to be relevant (Gordon et al., 2014). The households included in the present study typically relied on several different fuels, stoves, and locations for cooking, which may have varied according to changing needs or seasonal variations. Exposure to household air pollution induces inflammation of the respiratory tract and has been associated with pneumococcal colonization in epidemiological studies (Gordon et al., 2014; Rylance et al., 2016), as well as in experimental studies in vitro and in mice (Hussey et al., 2017). In the present study there was no significant association between pneumococcal carriage and the 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. It is therefore suggested that future studies include individual measurements of smoke exposure (Gordon et al., 2014).

Knowledge of antimicrobial use and susceptibility in human pathogens in Eastern Africa is limited. In this study, a significant increase in PNSP carried by children was found, 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 than that reported in pre-PCV13 studies in Tanzania, which found 69% non-susceptibility in colonizing pneumococci in Dar es Salaam and 68% in Moshi (Moyo et al., 2012; Bles et al., 2015). However, this is in line with the situation in Ghana after the introduction of PCV13 (Dayie et al., 2013). It is possible that penicillin non-susceptibility in healthy children in northern Tanzania was higher prior to the introduction of PCV13, as indicated in previous studies, and decreased as a result of vaccine introduction. However, the numbers of isolates in Dar es Salaam (Moyo et al., 2012) and Moshi (Bles et al., 2015) were small, and the sampled children in Moshi were born to HIV-positive mothers; thus this group was possibly more exposed to antibiotics due to increased sensitivity to infection. In addition, the present study sampling was started shortly after the introduction of PCV13, and a dramatic drop in non-susceptibility of carried pneumococci is less likely to occur so soon after vaccine implementation.

An 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 PNSP, as shown in the multivariable model. It appears that this has only been shown in one previous study conducted in Israel (Yagupsky et al., 1998), whilst several other studies have shown no association (Katsarolis et al., 2009; Stacevičienė et al., 2016; Samore et al., 2001; Moyo 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 this study, non-susceptibility to erythromycin was higher (15%) than that found in studies performed in Dar es Salaam and in Moshi in 2010, which reported 6.0% and 3.8% of non-susceptibility, respectively (Moyo et al., 2012; Bles et al., 2015). This implies an increase of almost four-fold 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 they are also effective against atypical agents such as *Mycoplasma pneumoniae* and *Legionella* spp. (Ministry of Health and Social Welfare, 2013). In the present study, erythromycin was the third most commonly used antibiotic in the children after amoxicillin and trimethoprim–sulfamethoxazole. In addition, high pneumococcal resistance to trimethoprim–sulfamethoxazole (97%) was found, which is similar to the findings of several studies performed in the region (Bles et al., 2015; Kobayashi et al., 2017; Moyo et al., 2012). This antibiotic was previously considered a first-line treatment for pneumonia, and usage of this antibiotic is still common for the 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 for whom antibiotics were prescribed for respiratory tract symptoms presented fast or laboured breathing, i.e. signs of pneumonia indicating the need for antibiotics, according to their parent or guardian. These results confirm previous findings of inappropriate prescribing practices by physicians in Moshi (Gwimile 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 a review of the child's medical log when possible. Effort was made to avoid confusion with other medicines by asking for the name of the antibiotic. Rates of antibiotic use based solely on parental recall are often underestimated, as shown in previous studies comparing parent-reported use of antibiotics with antibiotics detected in the urine of the child (Driscoll et al., 2012; Khennavong et al., 2011; Sombrero et al., 1999). Moreover, high use and misuse 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 this study, serotypes/groups 6, 19F, 23F, and 14 were the most prevalent serotypes/serogroups of those included in PCV13. These serotypes are considered colonizers 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 et al., 2018; Sleeman 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 introduction in both low- and high-income countries (Devine et al., 2017; Ho et al., 2015; Dube et al., 2018). Other studies in Africa have reported a high prevalence of serotypes/groups 15B/C, 10A, 11, and 19A (Birindwa et al., 2018; Kwambana-Adams et al., 2017; Dube et al., 2018). However, the relatively high prevalence of serotype 19B in this study was unexpected and appears not to have been reported in other recently published African studies.

Apart from protecting vulnerable subjects from invasive pneumococcal disease, PCV13 was also designed to target some serotypes more commonly associated with high non-susceptibility (Kyaw et al., 2006). Following the introduction of PCV, a change from PVC serotypes to non-PCV serotypes in the colonization of the nasopharynges has been shown (Croucher et al., 2013; Hammitt et al., 2014), which was also demonstrated in the present study. In the PCV era, colonizing pneumococci are thus exposed to selective pressure from both the vaccine and antibiotics administered. This may consequently select for those resistant strains not included in the vaccine (Danino et al., 2018; Croucher et al., 2014; Keenan et al., 2015). In line with this, the present study showed a notable increase in 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, with almost all being non-susceptible to penicillin (12 out of 13). Unfortunately, further analysis of possible clonality among these isolates could not be performed due to the scarce supply of genetic material.

In conclusion, penicillin non-susceptibility increased in colonizing *S. pneumoniae* in northern Tanzania during a 3-year period soon after the introduction of 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 effective treatment of pneumococcal disease.

Acknowledgements

Sincere thanks to Sister Celina Mayo and Sister Bertha Kiwale at the Community Health Department, KCMC for performing the parental interviews and sampling the children in the study. Our sincere appreciation also to all staff at the Clinical Laboratory, Microbiology Department at KCMC, especially Margaretha Sariko, Esther Kyara, Richard Tarimo, and Sophia Kombe for performing excellent laboratory work.

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 conducted 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.

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.

Funding

This study was supported by the Sahlgrenska Academy, University of Gothenburg and the Swedish International Development Cooperation Agency through provision of the Minor Field Study scholarship. The funding body had no role in the design of the study, the collection, analysis, interpretation of the data, or in the writing of the manuscript.

Conflict of interest

The authors declare that they have no competing interests.

Author contributions

SS, SEM, BN, and RA designed and sought ethical permission for the study. ME, JB, SF, and FJ together with local research nurses obtained consent from the parents or guardians to participate, acquired information for the questionnaires, and collected the samples. 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.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.ijid.2019.01.035.

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