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Research Article

A Comparative Study of Bacterial Isolates Cultured From the Nasopharynx of Children With and Without Sickle Cell Disease at a Tertiary Healthcare Institution in Nigeria

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- Sickle cell disease
- Healthy control
- MAR and virulence and resistance genes

Abstract

Background: Children with sickle cell disease have been shown to be at greater risk of bacterial infections from *Streptococcus pneumoniae*, *Hemophilus influenzae* and *Salmonella enteritidis* and from complications of septicaemia, pneumonia, respiratory difficulty and bone pain. The study compared the nasopharyngeal bacterial carriage of 87 children with sickle cell disease (SCD) and 160 children without SCD, ranging in age from four months to fifteen years. The study, conducted at the Wesley Guild Hospital, Ilesa in south- western Nigeria between January and November 2014, also looked at the antibiotic resistance profiles of the bacterial isolates and the presence of specific resistance and virulence genes.

Methods: Samples were collected from each study participant with the aid of a cotton-tipped applicator initially dipped into sterile saline and introduced into nasopharynx of subject, applied onto sterile thioglycolate fluid medium and incubated at 37°C for 24 hr. When growth was noticed, a loopful was retrieved and applied onto blood and chocolate agar as well as other selective and differential media. Bacterial colonies that grew on such media were picked and studied initially by Gram reaction, cultural and biochemical methods. Antibiotic susceptibility testing was done for selected bacterial isolatesalong with PCR demonstration of resistance and virulence genes.

Results: The results showed that the 1-5 and the 5-10 year SCD childrenhad the highest frequency of bone pain episode, hospitalization for malaria, bacterial infections and sepsis and blood transfusions for chronic anemia. *Corynebacterium* spp predominated among the nasopharyngeal isolates from both SCD and non-SCD children. C. *Xerosis* accounted for 55.38% among the non- SCD isolates and C. *Ulcerans* represented 35.84%of the SCD isolates.We recorded low carriage rates for *H. influenzae, S. Pneumonia* and *S.aureus* in the nasopharynx of the study participants. Widespread antibiotic resistance was observed among SCD and non-SCD isolates, with demonstration of resistance *blaZ* and *tetK resistance* genes and *sea*, and *eta* virulence genes by PCR.

Conclusion: The predominance in this study of *Corynebacterium* spp over organisms such as *H. influenzae,S. Pneumonia* and *S. aureus* that are common invasive pathogens of the nasopharynxwas both interesting and unexpected. The predominance suggested a shift in the community nasopharyngeal flora that may have resulted from long-term prophylactic use of antibiotics and successful administration of bacterial vaccines in the study population.

INTRODUCTION

Studies have shown that infants are more susceptible to infection because of their relatively immature immune system [1,2]. The nasopharynx of neonates is colonized early in life by bacterial organisms and with increasing frequency and diversity [3]. These organisms are initially acquiredat birth perinatally and postnatally from their mothers and other handlers [4]. The resulting nasopharyngeal flora often includes potential pathogens such as *Streptococcus pneumoniae*, *Hemophilusinfluenzae* and *Neisseria meningitidis* which maycause pneumonia and meningitis [3,4]. The threeorganisms are also a major cause of childhood

morbidity and mortality in young children.A study by the World Health Organization (WHO) reported that pneumococcal disease is responsible for an average of 454,000 deaths annually among children <5 years of age [5]. But the introduction of viable vaccines such as the PCV 7 and 13 and the Hib conjugate vaccines has significantly reduced the development of early childhood diseases in developed and developing countries [6-8].

Sickle cell disease is a major hereditary disease with a high prevalence rate in sub-Saharan Africawhere more than 230,000 children are born annually with sickle cell disease [9-11]. An estimated 150,000 children are also born with sickle cell trait (HbAS) annually in Nigeria, with one in four Nigerians carrying the trait. On the other hand, only one in 12 African Americans carries the trait. Although over 700 structural haemoglobin (Hb) variants have been identified worldwide, only HbS and HbC predominate in Africa [12]. Sickle cell disease(SCD) predisposes the host to a number of complications that include septicaemia from infections, chronic anemia, leg ulcers, spleen atrophy and chronic bone pain as well as immune dysregulation [13].We therefore designed our study to determine and compare the pattern of nasopharyngeal bacterial carriage between a cohort of SCD children and children without sickle cell disease who attended the Child Health Clinic of the Wesley Guild Hospital at Ilesa in south-western Nigeria between January and November 2014. The study also compared the antibiotic susceptibility profiles of the dominant bacterial isolates from the two cohorts. Information on haemoglobin genotypes and severity of disease among the SCD children was obtained from medical records and questionnaire interviews of patients and their parents. We also characterized the antibiotic resistanceand virulence genes of thenasopharyngeal isolates of *Staphylococcus aureus* from the two study cohorts. It is expected that the results obtained from this study would clarify differences in the nasopharyngeal floras of SCD children and non-SCD children as well as suggest improvement in clinical management of associated bacterial infections.

METHODS

Study center and inclusion criteria

The study was conducted at the Wesley Guild Hospital at Ilesa in south western Nigeria. The hospital is a satellite center of the Obafemi Awolowo University Hospitals Complex at Ile-Ife, Nigeria. Ilesa is a town of about 277,904 inhabitants and is located at approximately 25 miles from Ile-Ife.Approval for the study was obtained from the Human Subject and Ethical Review Committees of the hospital. Two study cohorts comprising of children with SCD and those without SCD were selectedfrom among young patients who attended the Child Health Clinic of the Ilesa hospital. While participation in the study was voluntary, subjects were recruited after careful explanation of the aims of the study and obtaining informed consent from the parents or guardians.

A total of 87 SCD children and 160 non-SCD children were enrolled in the study. They ranged in age from 4 months to 15 years. Seventy eight (92.85%) of the SCD children were genotyped as HbSS and six (7.24%) as HbSC. The SCD group included 45 (54%) males and 39 (46%) females. Thenon-SCD children included 77 (48%) males and 83 (52%) females. Forty two (42) of the non-SCD children were of HbAS genotype. Information relating to severity of disease in SCD children such as the number of pain episodes, hospitalizations and blood transfusions was extracted from patient medical records.

Sample collection and culture isolate identification

Nasopharyngeal culture samples were obtained from the study subjects by an attending physician using a sterile cotton-tipped applicator that was initially dipped in sterile saline. Samples wereinoculated into duplicate sterile thioglycollate fluid mediaand separately incubated at 37°C aerobically and in anaerobic jarsfor 48hrs.For biochemical characteristics and identification, aloopful of all cultures was streaked onto blood agar (BA), chocolate agar (CA), mannitol salt agar (MSA), eosin methylene blue agar (EMB), sulfide indole motility agar (SIM), Simmon citrate agar and triple sugar iron agar (TSI) (Oxoid LTD, Basingstoke, Hampshire, England). The plates were incubated aerobically and anaerobically at 37° C for 48 hr. Colonies from each culture mediumwere Gram stained and processed for biochemical identification using the Analytical Profile Index (API) 20E and API Staph (Biomerieux, France).Coagulase and catalase tests and sensitivity to Taxo A disc and Taxo P disc(BD Diagnostics, Difco Laboratories, Detriot, USA) were also employed for identification. Antibiotic susceptibility tests were carried out by the Kirby Bauer disc diffusion method using the Mueller Hinton agar. The antibiotics included erythromycin (15 μg), gentamicin (10 μg), agumentin (30 μg), streptomycin (10 μg), tetracycline (10 μg), chloramphenicol (10 μg), nalidixic acid (30 μg), ampicillin(10 μg), nitrofurantoin (200 μg), ciprofloxacin (5 μg), ceftriaxone (30 μg), oxacillin (1 μg) and kanamycin (30 μg). *S. aureus* ATCC 25923 and *Enterobacter aerogenes* (American Type Culture Collection, Rockville, USA) were used as control organisms.

DNA extraction of S. aureus isolates

The DNA of each *S. aureus*isolate was extracted by suspending the bacterial the colony in 200μl of sterile distilled water in labelled eppendorf tube and centrifuged at 13,000rpm for 3mins. The suspension was heated at 100°C for 10 minutes in a microwave machine (Haier thermocool), cold shocked in ice for 2 mins and vortexed (Gallenkamp spin-mix, Germany), re-centrifuged at 10,000 rpm for 1 min and then stored at -20°C for DNA amplification [14].

PCR detection of S. aureus nuc, bla Z, tet K, sea and eta genes

Multiple antibiotic resistant *S. aureus*isolates are known to carry *nuc*, *bla Z* and *tet K* resistancegenes as well as the *sea* and *eta virulence* genes that code for production of enterotoxin A and exfoliative toxin A respectively[15]. Details of specific primers that were used to amplify sequences of the virulence and resistance genes as well as thepredicted sizes of the amplified products and specific annealing temperatures are given in Table 2.

A 25μl mixture containing 4μl of 10X buffer, 0.5μl $MgCl_2$, 3μl dNTPs, 0.2μl Taq polymerase, 1μl of the forward primer, 1μl of the reverse primer and 5μl of extracted DNAwas prepared in

a PCR vial. The vial was placed in a programmed thermocycler (iCycler; Bio-Rad, Milan, Italy) with an initial denaturation at 94°C for 5 minutes, followed by 30 cycles of denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute and extension at 72°C for 1 minute. A final extension procedure was carried out at 72°C for 10 minutes.

Agarose gel electrophoresis

Amplified PCR products were electrophoresed on 1% agarose gel containing 0.5 µl of ethidium bromide and run through 100 volt for 25 minutes in 1X TBE buffer in the electrophoresis tank. For each run, a 100 base-pair molecular weight DNA standard (size marker) was used to verify the appropriate size of each amplified PCR product. The DNA bands were then captured and visualized with a short wave ultraviolet transilluminator (UV Transilluminator 2000; Bio-Rad, Milan, Italy) and photographed using a Kodak digital camera.

RESULTS

The study included 87 SCD children and 160 non-SCD children, all ranging in age from 4 months to 15 years. Our results show that 29 (34.5%) of the SCD children were 1-5 years old, 35 (41.6) were 5-10 years of age and 18 (21.4%) were 10-15 years old (Table 1). The 1-5 and the 5-10 year old groups had the highest frequencies of bone pain episode, hospitalization for malaria, bacterial infections and sepsis and blood transfusions for anemia. On the other hand, 60 (37.5%) of the 160 non-SCD children were diagnosed with a variety of ailments that included tonsillitis, pharyngitis, whooping cough, diarrhea, otitis media, pneumonia, maculo-papula rash, tineacapitis, sepsis, impetigo, skin lesions , asthma , conjunctivitis, mumps, stomatitis and cerebral palsy.

Table 2 and Figure 1 show the distribution of the bacterial isolates that were cultured from the nasopharynx of SCD, non-

Table 1: Indices of disease severity in children with sickle cell disease.

Number of subjects in different age groups with				
Age group	**Pain episodes**	**Hospitalizations**	**Blood transfusions**	**Total No. Subjects**
0 – 11 mths	2	1	1	2 (2.4%)
1-5 yrs	23	19	11	29 (34.5%)
5-10 yrs	29	22	11	35 (41.6%)
10-15 yrs	7	7	7	18 (21.4%)
Total	**61**	**49**	**30**	**84* (100%)**

Table 2: Distribution of bacterial isolates cultured from the nasopharynx of children with and without sickle cell disease.

	SCD (No. %)	Non SCD (No. %)
Gram positives		
Corynebacterium spp		
C. xerosis	19 (24%)	48 (29%)
C. diphtheriae	11 (14%)	10 (6%)
C. pseudodiphtheriticum	10 (12%)	0 (0%)
C. ulcerans	8 (10%)	72(43%)
Other gram positives		
Arcanobacterium	6 (7%)	1 (0.9%)
Haemolyticum		
Bacillus subtilis	3 (4%)	14 (8%)
Staphylococcus aureus	4 (5%)	18 (11%)
Staphylococcus spp	5 (6%)	3 (2%)
Streptococcus	3 (4%)	0 (0%)
Pneumonia		
Sarcina spp	5 (6%)	0 (0%)
Actinomyces isrealii	6 (7%)	0 (0%)
Nocardiaasteroides	1(1%)	0(0%)
Total	**81 (100%)**	**167 (100%)**
Gram negatives		
Moraxella catarrhalis	2 (6%)	18 (32%)
Haemophilus influenzae	5(14%)	33 (59%)
Klebsiella pneumoniae	3 (8%)	5 (9%)
Pseuomonas aeruginosa	7 (19%)	0 (0%)
Salmonella enteritidis	3 (8%)	0 (0%)
Other gram negatives	10 (28%)	0 (0%)
Total	36 (100%)	56 (100%)

SCD and control children. The gram positives represented about 72% of the total341bacterial isolates compared to 28% for gram negatives. Interestingly, the *Corynebacterium* species represented 64% and 85% of the bacterial isolates from the nasopharynx of SCD and non-SCD childrenrespectively. *Streptococcus pneumoniae*, though at a low frequency, was only isolated from SCD children, while *Staphylococcus aureus* and *Bacillus subtilis* were more predominant in the non-SCD children than in the SCD children.

Of the 119 bacterial isolates that were cultured from theSCD children,53 (44.5%) were *Corynebacterium* species that included*C. xerosis* (35.8%),*C. diphtheriae*(20.75%) and *C. pseudodiphtheriticum* (15.1%). Furthermore, 66(55.46%) of the bacterial isolates from SCD children were gram positive rods.*S. aureus*and *S. pneumoniae*represented 33% and 25% of the 124 gram positive coccal isolates.Also 41(34.45%) bacterial isolates that were recovered from the nasopharynx of SCD children were gram negative rods.*Pseudomonas aeruginosa* accounted for 7(17.07%)while14.6% and 12.2% *were Streptobacillus moniliformis*and *Haemophilus influenzae* respectively. *Klebsiella pneumoniae, and Salmonella enteritidis*and *Moraxella catarrhalis* were less than 5% each.Out of the 222 isolates cultured from non-SCD subjects, 145 (65.31%) were gram positive bacilli, 56(25.22%) were gram negative rods (Table 2). *Corynebacterium* spp constituted the predominant gram positive organisms at 58.55%, with *C. ulcerans* at 55.38%, *C. xerosis* (36.92%) and *C. diphtheriae*at 7.69%. Of the 56 gram negative bacilli cultured from the nasopharynx of non-SCD children, 33(58.92%) were *Haemophilus influenzae*, 18(32.14%) were*Moraxella catarrhalis* and 5(8.92%) were *Klebsiella pneumoniae*.

The antibiotic resistance profiles of *S. aureus* isolates that were cultured from the nasopharynxof SCD, non-SCD and control children were also determined. Of the 18 *S. aureus* isolates from the non-SCD children, 14were resistant to ampicillin, 11to erythromycin, 9 to tetracycline, 7 to ceftriaxone, 2 to chloramphenicol, 1 to streptomycin, kanamycin and ciprofloxacin each. All isolates were sensitive to augmentin, oxacillin, gentamycin and nitrofurantoin. In contrast, the three *S. aureus* isolates from the SCD children were resistant to ampicillin, erythromycin and nalidixic acid. PCR was used to detect the presence of *nuc, bla Z, tet K, sea* and *eta* genes in eleven methicillin sensitive*S. aureus* isolates. The *nuc, blaZ* and *tetK*genes code for antibiotic resistance while the *sea* and *eta* genes code for enterotoxin A and exfoliative toxin A respectively. Table 3 shows a list of the primers that were used to amplify sequences of the virulence and resistance genes as well as the predicted sizes of the amplified products and specific annealing temperatures. Amplified products were electrophoresed on 1% agarose gel containing 0.5 µl of ethidium bromide, along with molecular weight DNA standard size markers to verify the appropriate size of each amplified PCR product. The results, presented in Table 3 and Figures 2, show that all eleven isolates carried the *nuc* gene while nine isolates carried the *blaZ* gene. Five isolates carried the *sea* gene, four carried the *tetK* gene and three isolates carried the *eta* gene. One isolate (F9ma) carried all five genes, two isolates (A2m2, B2mi) carried four of the 5 genes, and four isolates (Al16B1, D11Cm, 13ma, l18mb) carried three of the genes while three isolates only carried two of the genes.

DISCUSSION

Studies have shown that bacteria organisms of the nasopharyngeal flora of SCD children may become invasive and establish blood and lung infections. We sought to investigate whether there were differences in the nasopharyngeal flora and carriage rates of children with SCD and without SCD and in the antibiotic resistance profiles of the bacterial isolates. Studies already showed that children with SCD are significantly more susceptible to infections from *S. pneumoniae*, *H. influenzae*,*S. enteritidis*, malaria and other microbial organisms [11]. Our study included 87children with SCD and 160 children without SCD who attended theWesley Guild Hospital at Ilesa in south-western Nigeria and who ranged in age from 4 months to 15 years. Our data show the indices of disease severity in the SCD children as

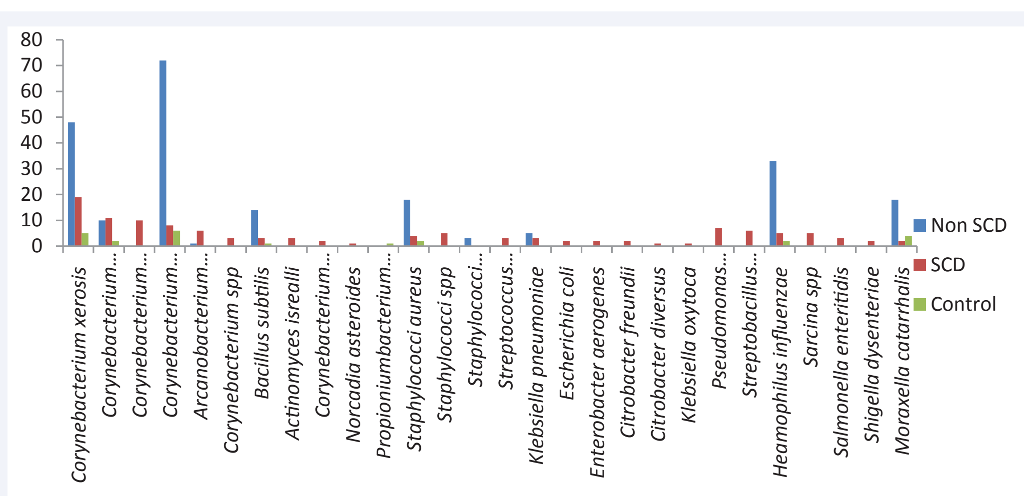

Figure 1 Shows the frequency distribution of bacterial isolates cultured from the nasopharynx of Non SCD, SCD and control subjects.

Frequency of Bacterial isolates cultured from the nasopharynx of Non- SCD, SCD and Controls, Non SCD = Sick children without sickle cell disease; SCD = Children with sickle cell disease; Control = apparently healthy

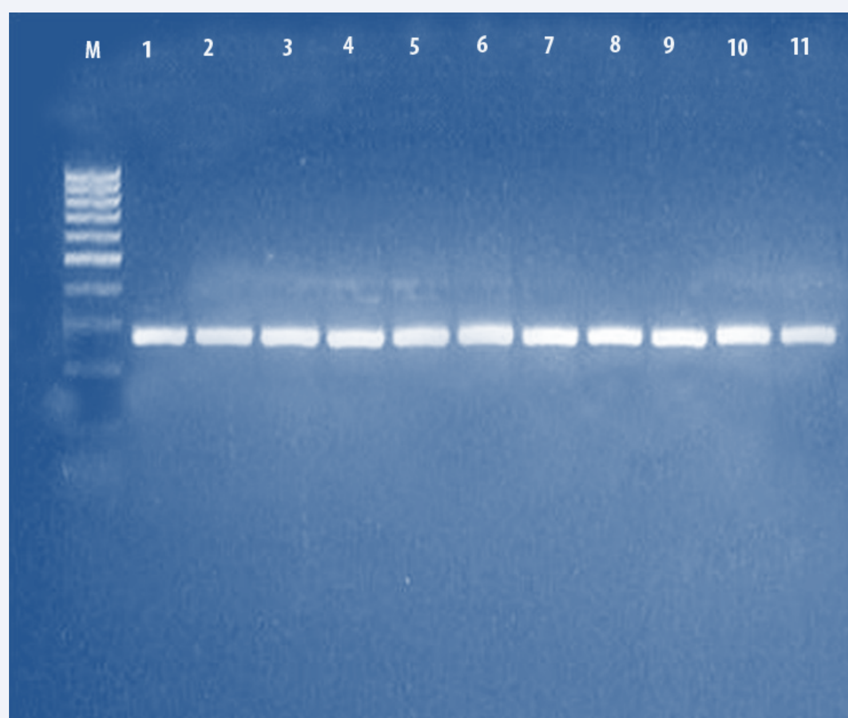

Figure 2 PCR Identification of the nuc gene (270 bp); M – 100 bp size marker, 1-11 samples.

Table 3: Primers used for the Detection of Resistance and Virulence genes.

Target Genes	Oligonucleotide Sequence (5' – 3')	Size of Target Region (bp)	Annealing temperature (°C)	Reference
Nuc	F- GCGATTGATGGTGATACGGTT R- AGCCAAGCCTTGAACGAACTAAAGC	270	55	[19]
Tet K	F- GTAGCGACAATAGGTAATAGT R- GTAGTGACAATAAACCTCCTA	360	55	[20]
Bla Z	F- ACTTCAACACCTGCTGCTTTC R- TGACCACTTTTATCAGCAACC	172	55	[21]
Sea	F- GGTTATCAATGTGCGGGTGG R- CGGCACTTTTTTCTCTTCGG	102	60	[15]
Eta	F- GCAGGTGTTGATTTAGCATT R- AGATGTCCCTATTTTTGCTG	93	56	[15]

indicated by the frequency of bone pain episodes, hospitalizations and blood transfusions. The results also show a high frequency of bone pain episode, hospitalization for malaria, bacterial infections and sepsis, and blood transfusions for chronic anemia among the 1-5 and the 5-10 year old SCD children. On the other hand, clinical records showed that 60 (37.5%) of the 160non-SCD children had acute presentations of non-fatal illnesses such as tonsillitis, pharyngitis, whooping cough, diarrhea, otitis media, maculo-papula rash and others. The results are readily explained by the immune dysregulationprocesess that characterize the pathogenesis of SCD.

Information on the distribution of bacterial isolates from the nasopharynx of children with and without SCD is presented in Table 2. Overall, 364 bacterial isolates were cultured from 270 subjects that were recruited for the study. A total of 119 isolates were cultured from 87 sickle cell disease children while 222 bacterial isolates were cultured from 160 children without sickle cell disease.*Corynebacterium* species were the most prevalent bacteria among the three cohort samples.*C. xerosis* was the dominant species among the SCD children, while *C. ulcerans*was isolated most frequently amongthe non- SCD and control subjects.

The predominance in this study of *Corynebacterium* species over organisms as *H. influenzae,S. pneumoniae*and *S. Aureus* that are common invasive pathogens of the nasopharynx was interesting and unexpected. Our results also show that while the prevalence of H. *influenzae* 33(14.8%) is relatively high among children without SCD in this study, the prevalence was remarkably low in children with sickle cell disease (4.2%) and control subjects (8.6%). Similar findings have been reported by other investigators [16]. The study found that high carriage rate of *S. pneumoniae* did not correlate with invasive bacteremia. In our study, the prevalence of nasopharyngeal carriage with both *H. influenzae* and *S. pneumoniae* was similarly low for SCD children compared to children without SCD (Table 2). While the reason

Table 4: Characterization of the methicillin sensitive *S. aureus* (MSSA) obtained from the nasopharynx of non -SCD in Ilesa.

Isolate code	Location	Clinical Diagnosis	Antibiogram	*nuc* gene	blaZ gene	tetK gene	*sea gene*	*eta gene*
A2m2	Ilesa	Uncomplicated malaria	TET, AMP & ERY.	+	+	+	-	+
Al16B1	Ilesa	Enlarged tonsils	AMP, ERY & CIP	+	+	-	+	-
B2m1	Ilesa	Uncomplicated malaria	TET, AMP, KAN, ERY &CRO	+	+	+	-	+
B5C1	Ilesa	Uncomplicated malaria	TET, AMP & STREP	+	+	-	-	-
D11Cm	Ilesa	Uncomplicated malaria	AMP, ERY & CRO	+	+	-	+	-
E22Ba	Ilesa	Uncomplicated malaria/ Tonsilitis	TET, AMP, ERY & CRO.	+	+	-	-	-
F9ma	Ilesa	Uncomplicated malaria	TET, AMP, AUG, ERY, CRO	+	+	+	+	+
F9mb	Ilesa	Uncomplicated malaria	TET, AMP, ERY, CRO	+	-	-	-	-
G3ma	Ilesa	Tonsilitis/ uncomplicated malaria	TET, AMP, ERY	+	-	+	-	-
I3ma	Ilesa	Vaso-Occlusive crisis	TET, AMP, ERY	+	+	-	+	-
I18mb	Ilesa	Sore throat	AMP, CHL, CRO	+	+	-	+	-
I1b	Ilesa	Control	0	NA	NA	NA	NA	NA
I8m	Ilesa	Sore throat	CHL,CRO	NA	NA	NA	NA	NA
F6mb	Ilesa	Uncomplicated malaria	0	NA	NA	NA	NA	NA
F29mc	Ilesa	Uncomplicated malaria	0	NA	NA	NA	NA	NA
F4ma	Ilesa	Uncomplicated malaria	AMP,CRO	NA	NA	NA	NA	NA
F3m	Ilesa	Uncomplicated malaria	0	NA	NA	NA	NA	NA
I9m	Ilesa	Diarrhoea	CHL, ERY	NA	NA	NA	NA	NA
G8m	Ilesa	Bone pain crisis	AMP	NA	NA	NA	NA	NA
G22m	Ilesa	Pharyngitis	AMP, ERY	NA	NA	NA	NA	NA

Legend: + = positive, - = negative, NA= Not applicable

for low incidence is not apparently clear, the observation may be attributed to long-term prophylactic treatment of children with penicillin and other antibiotics and theeffectiveness of the administration of pneumococcal and Hib vaccines to the study cohorts from early age at the hospital clinic. It is notable from the results that while the prevalence of *S. pneumoniae* and *H. influenzae* in the nasopharynx of the study population has been significantly reduced by penicillin and vaccine administration, the two organisms have largely been replaced by various species of *Corynebacterium*, which are opportunist respiratory pathogens that can cause diphtheria and pharyngitis.

Studies reported from Ile-Ife in the 1990s showeda high prevalence of methicillin resistant *S. aureus* (MRSA) strains in the anterior nares. However, our present observation is at variance with such previous findings [17]. The results from our present study show that all *S. aureus*isolates that were recovered from the nasopharynx of the children were methicillin sensitive strains (MSSA). This is an interesting shift of community bacterial flora since the past two decades. Other investigators have reported a similar reduction in the prevalence of MRSA isolatesfrom non-SCD children [18]. Our investigation also revealed that eleven *S. aureus* isolates from non -SCD children carried resistance and virulence genes. Nine of the 11 *S.aureus* isolates from the nasopharynx of non- SCD children carried the *blaZ* resistance gene which confers resistance to the beta- lactam antibiotics. Four isolates carried the *tetK* virulence gene which confers resistance to tetracycline. Five isolates carried the *sea* virulence gene that codes for enterotoxins while three of the 11isolates carried the *eta* virulence gene that codes for the staphyloccocal scalded skin syndrome toxin [15]. This is the first report that has documented the presence of those genes in *S. aureus* isolates in the study area and the results are significant because of the diverse and differential carriage of both antibiotic resistance genes as well as the virulence genes. In patients with inadequate immunity as in SCD children, the dissemination of such resistant and potentially virulent organisms from the nasopharynx could result in invasive bacteremia, intravascular coagulation and septicaemia [13].

In conclusion the bacterial isolates cultured from the nasopharynx of SCD, non-SCD and control subjects were predominantly *Corynebacterium* spp. that were sensitive to all the antibiotics used in the study. We examined eleven *S. aureus* isolatesfor their carriage of antibiotic resistance and virulence genes and found that these genes are present in most of the isolates. Our study revealed low incidence of nasopharyngeal colonization with *both H. influenzae* and *Streptococcus pneumoniae* in this centre but the widespread antibiotic resistance among *S. aureus* isolates among sick children portends serious challenges for therapeutic options.The results of the study suggest the need for a concerted educational program in the appropriate use of prophylactic antibiotics and of an expanded immunization program with coverage for all children.

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