A study of the intestinal carriage of antibiotic resistant *Staphylococcus aureus* by Nigerian children

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Abstract

Background: The gastrointestinal tract has been recognized as a major ecological site for *Staphylococcus aureus* where it can reach neighboring sites and cause mild or serious infections.

Objectives: To determine the prevalence of intestinal carriage of *S. aureus* in children aged 3 years and below in Ile-Ife, Nigeria and the antibiotic resistance characteristics of the organisms obtained.

Methods: The organisms isolated in the course of the study were identified by phenotypic and genotypic methods and screened against 13 antibiotics by conventional methods. A total of 293 subjects were sampled of which 130 were diagnosed with diarrheal at the time of the study while the rest were apparently healthy.

Results: 14.0% of the faecal samples yielded *S. aureus* with the carriage rate among the subjects being found to be highest at about 1 month approximately in subjected ages. Sixty-five percent of the isolates were found to be resistant to more than three different antibiotics with more than 50% being resistant to penicillin, erythromycin and trimethoprim.

Conclusions: The results of the study indicated that a reservoir of multiply antibiotic resistant *S. aureus* exists in the gastrointestinal tracts of children living within the study environment.

Keywords: *S. aureus*, faecal carriage, oxacillin resistance, antibiotic resistance, children, Nigeria.

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Introduction

Staphylococcus aureus is a gram positive bacteria that occurs naturally in and on the human body. It is also an important opportunistic pathogen responsible for a variety of diseases, ranging from minor skin infections such as styles, furunculosis and paronychia to life threatening systemic infections such as pneumonia, endocarditis and sepsis[1]. The versatility of *S. aureus* contributes to the impressive capacity of the pathogen to colonize and persist in a range of diverse environments, it being a persistent colonizer of the anterior nares in 20% of the population and is intermittently carried by another 30% [2]. The organism can also be detected in other moist regions of the human body, such as the throat, axillae, vagina and the intestinal tract and thereby has several niches in the body which form major reservoirs for infection[1].

Colonisation by *S. aureus* is a major risk factor for staphylococcal infections as it has been shown, for example, that 80% of nosocomial *S. aureus* bacteremia cases have an endogenous origin[1,2].

The human intestinal tract harbors a large, active and complex community of microbes of more than 500 different microorganisms' species. With 10^{12} bacteria per gram of faeces, the colon, in particular, is confronted with the highest bacterial load[3,4]. Gram negative bacteria of the family enterobacteriaceae such as *Escherichia coli, Enterococci, Enterobacter,* and *Klebsiella* and anaerobic bacteria such as those of *Bifidobacterium, Clostridium* and *Bacteroides* genera, staphylococci such as *S. aureus* and a wide variety of other culturable and non-culturable bacteria exists as part of the normal flora in the intestinal tract and can be found in infant stools[3,4].

Colonization of the gastrointestinal tract by *S. aureus* has been well documented in literature [5] and rectal and perineal carriages of *S. aureus* have also been documented as potential sources of both endogenous and exogenous staphylococcal infections[3]. Faeces can certainly be important as a source of environmental contamination and has been identified as a possible source of antibiotic resistant *S. aureus*[4]. In addition to this, perineal carriers have been reported to disperse more *S. aureus* into the environment than nasal carriers[5].

This study attempts to provide information about some of the characteristics associated with

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the carriage of *S. aureus* in the gastrointestinal tracts of Nigerian children. The aim of this study, therefore, is to determine the prevalence of intestinal carriage of *S. aureus* in children aged 3 years and below in Ile-Ife, Nigeria and the antibiotic resistance characteristics of the organisms obtained.

Methods

Setting and study population

The study was carried out in Ile-Ife, a semi-urban town in Osun State of Nigeria. Samples were also collected from children in villages around Ile-Ife.

The study was primarily concerned with studying the prevalence of *S. aureus* in the intestinal tract of children aged below three years and children with diarrhea and apparently healthy children were investigated. The children with diarrhea were those who passed frequent watery stools, at least three times a day or were diagnosed as such by a physician while the apparently healthy children were those with no fever, no diarrhoea (as defined), not on hospital admission and with no history of chronic illness. All subjects were reported not to have taken any antimicrobial agent in the last two weeks preceding sampling. Data about children were collected through the parents or guardians of the child.The children were among those presenting for immunisation and treatment at five different community health centres and children attending four day-care centres including those from households selected randomly in the Ile-Ife community.

To get the appropriate number of subjects required to draw valid conclusions, sample size determination was done using the formula for sample size calculation as described previously[6,7].

The study was approved by the institutional review board of each of the participating institutions and all specimens were collected with the informed consent of the parents or guardians of the children.

Sample collection and isolation of *S. aureus*

Freshly voided stools were collected over a period of six months (January to June, 2006) from a total of 293 children. The specimens collected were plated on mannitol salt agar and incubated at 37^{o}C for 24 - 48 hours. Pure colonies obtained were then subcultured onto fresh mannitol salt agar and blood agar. *S. aureus* were identified by colonial characteristics on blood agar and mannitol salt agar, by gram stain reactions, and by biochemical tests including catalase test, modified oxidase test, alkaline phosphatase test, and slide and tube coagulase test. The colony characteristics on the media were noted. *S.aureus* NCTC 6571 was used in all experimental work as control. *S. aureus* (MRSA) ATCC ^{R} 43300 was used for the polymerase chain reaction analysis as positive control while sterile distilled water was used as negative control.

Antibiotic susceptibility testing

The standard disc diffusion method approved by the Clinical Laboratory Standard Institute (CLSI) was employed[8]. The susceptibilities of the *S. aureus* isolates were tested using the high (equivalent to 0.5 McFarland standard) density inocula prepared as described[9] . The following antimicrobial agents at the indicated concentration were tested: clindamycin (CM) 15μg/disc, chloramphenicol (CL) 30μg/disc, trimethoprim (TR) 5μg/disc, fusidic acid (FU) 50μg/disc, ciprofloxacin (CI) 10μg/disc, penicillin V (PV) 10μg/disc, erythromycin (EM) 15μg/disc, tobramycin (TM) 30μg/disc, obtained from AB-Biodisc, Sweden; amikacin (AKN) 10μg/disc obtained from Institute Pasteur, France; tetracycline (TET) 30μg/disc, co-trimoxazole (COT) 25μg/disc, gentamicin (GEN) 30μg/disc obtained from Abtek, England and oxacillin (OX) 1μg/disc obtained from Oxoid, England. The diameters of inhibition zones were measured in millimeters and interpretation was done using the Progressive Diagnostics Manufacturers (PDM) Interpretive Charts (AB Biodisc, Sweden) which agrees with the CLSI requirements[8].

Determination of oxacillin susceptibility

Agar screening method

Oxacillin-salt agar (Mueller-Hinton agar containing 4% NaCl and 6 mg oxacillin L^{-1}), was used for the agar screening method as recommended by the CLSI[8]. Inoculation of the oxacillin-salt agar plates was performed by using a multipoint inoculator. Any isolate which grow on the test medium within 48 hours was regarded as being oxacillin resistant. The experiment was performed in duplicate.

Molecular confirmation of *S. aureus*

S. aureus isolates were confirmed as *S. aureus* by polymerase chain reaction (PCR) amplification of thermostable nuclease gene (*nuc*) using the primers, *nuc*-F (5'GCGATTGATGGTGATACGGTT-3') and *nuc*-R (5'-AGCCAAGCCTTGACGAACTAAAGC-3') with the PCR conditions previously described[10].

Molecular detection of the *mecA* gene by PCR

The presence of the *mecA* gene responsible for the production of PBP2A was determined in all *S. aureus* isolates resistant to oxacillin by the disc diffusion and agar-screening techniques.

The primers for the *mecA* gene were as previously described[11]. The primers were mecA-1 (5'-AAAATCGATGGTAAAGGTTGGC-3') and mecA-2 (5'-AGTTCTGCAGTACCGGATTTGC-3'). *S. aureus* (MRSA) ATCC R 43300 was used for the PCR analysis as positive control for the *mecA* gene while sterile distilled water was used as negative control.

PCR conditions for amplification of the *mecA* gene comprised pre-denaturation, 94°C for 3 minutes, followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 30 seconds, followed by final extension for 4 minutes at 72°C.

The samples were electrophoresed for 60 min at 80V. A 100-bp DNA Molecular Weight Marker XIV (Roche Diagnostics GmbH, Germany) was used as a molecular size marker in each gel. DNA fragments of 532bp which corresponded to the *mecA* PCR products were visualized on a UV trans-illuminator at 320nm after staining with ethidium bromide (1mg/L) for 15 minutes and destaining in distilled water for 30 minutes.

Statistical analysis

Chi-square test or the Fisher exact test was used in determining probabilities and level of significance. It was hypothesised that the age, gender and the health status of children will influence the prevalence of faecal carriage of *S. aureus*. All hypotheses were considered significant if $p<0.05$. The analysis was performed using SPSS Version 12 statistical software.

Results

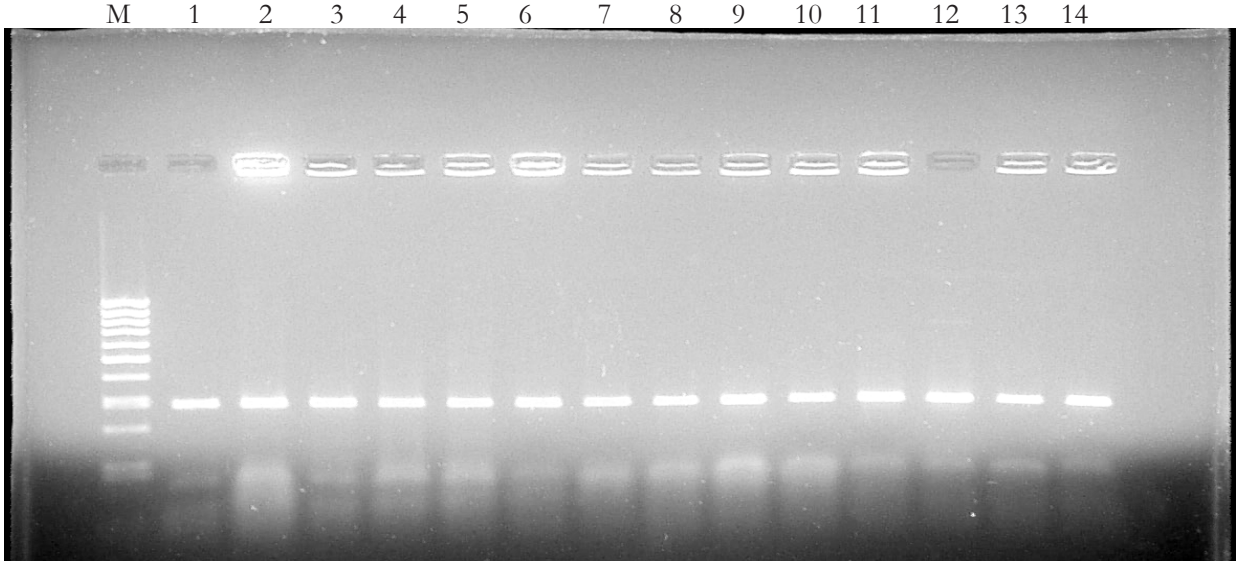
Carriage of *S. aureus* by children

The result (Table 1) shows that *S. aureus* (figure 1) was isolated from 41 (14.0%) of the 293 children. Of the 41children colonized with *S. aureus*, 23(17.2%) of the children were of age group 0-6 months, 11 (16.9%) of age group 7-12 months and 7 (11.1%) of age group 13-24 months. Although the incidence of *S. aureus* tended to decrease with age, this tendency was not statistically significant ($p = 0.330$). None of the children above 2 years old had *S. aureus* recovered from them. There was no significant relationship ($p>0.05$) between *S. aureus* faecal carriage and children characteristics such as the gender of the children and whether the child had diarrhoea at the time of collection or not.

Table 1: Fecal carriage of *S. aureus* according to age groups

Age group, months (No)	MSSA No (%)	MRSA No (%)	Total No (%)
0-6 (134)	13 (9.7)	10 (7.5)	23 (17.2)
7-12 (65)	8 (12.3)	3 (4.6)	11 (16.9)
13-24 (63)	4 (6.3)	3 (4.8)	7 (11.1)
25-36 (31)	0	0	0
Total (293)	**25 (8.5)**	**16 (5.5)**	**41 (14.0)**

Figure 1: PCR amplification of the *nuc* gene



M is the 100bp marker, Lane 1 is the positive control, Lane 2 to 14 are *S. aureus* test strains

Antibiotic resistances in *S. aureus* isolates

The result of antibiotic resistance in the *S. aureus* isolates is shown in table 2. Susceptibility of the *S. aureus* strains to gentamicin was 100% while only 7.3% and 12.2% of the isolates were resistant to clindamycin and the fluoroquinolone, ciprofloxacin respectively.

Table 3 shows the antibiotic resistance phenotypes of the isolates. Thirty one different phenotypes were obtained out of which 23(74.2%) isolates had 23 different phenotypes, 12 isolates has six different phenotypes while the remaining six isolates had two different phenotypes. Resistant rates ranged from 1 to 10 of the 13 antibiotics tested. Twenty-seven (65.9%) of the 41 *S. aureus* isolates were simultaneously resistant to more than three of the antibiotics tested against them. Three of the organisms did not show resistance to any of the agents.

Table 2: Antibiotic resistance profile of *S. aureus* isolates

Antibiotics	CM	TET	COT	CL	GEN	TR	FU	CI	PV	EM	TM	AKN	SU	OX
% Resistance	7.3	39.0	46.3	39.0	0	80.5	22.0	12.2	90.2	65.9	14.6	14.6	31.7	39.0

Table 3: Antibiotic resistance phenotypes in the *S. aureus* isolates

Serial No	Resistance phenotpes	Frequency of occurrence
1	NO RESISTANCE	3
2	PV	1
3	PV, TR	3
4	PV, EM	1
5	COT, TR	1
6	PV, OX	1
7	PV, EM, TR	1
8	PV, CL, TR	2
9	PV, TET, TR	1
10	PV, EM, COT, TR	1
11	PV, CL, OX, TR	1
12	PV, CL, FU, TR	1
13	PV, CL, EM, TR	1
14	PV, EM, COT, TR	1
15	PV, TET, EM, COT	1
16	PV, EM, COT, CI, TR	2
17	PV, TET, EM, CL, TR	2
18	PV, EM, COT, OX, TR	2
19	PV, TET, EM, CL, COT, TR	1
20	PV, TET, EM, CL, OX, TR	1
21	PV, EM, COT, FU, OX, AKN	1
22	PV, EM, COT, FU, OX, TR	1
23	PV, TET, EM, COT, FU, OX, TR	1
24	PV, TET, EM, CL, COT, TR, AKN	1
25	PV, EM, CL, FU, CI, OX, TR	1
26	PV, TET, EM, COT, CI, OX, TR, AKN	1
27	PV, TET, EM, CL, COT, OX, TR, CM	1
28	PV, TET, EM, CL, FU, TM, OX, TR	2
29	PV, TET, EM, COT, FU, TM, TR, CM, AKN	1
30	PV, TET, EM, CL, COT, TM, OX, TR, AKN	2
31	PV, TET, EM, COT, FU, TM, CI, OX, TR, CM	1

Carriage of MRSA by children

Of the 41 *S. aureus* isolated from the children 16(39.0%) were found to be oxacillin resistant. The disc-diffusion test as well as the oxacillin agar plate screening method gave the same results and these were confirmed by the detection of the *mecA* gene by the polymerase chain reaction.

There was no relationship ($p>0.05$) between oxacillin resistance rates and the gender of the children, whether the child had diarrhoea at the time of collection or not, and whether the child was from day care or health centres.

Discussion

The carriage and pattern of prevalence of *S. aureus* in the gastrointestinal tract of children in Ile-Ife and environs were investigated. Results indicate that 14.0% of the children had *S. aureus* isolated from their faecal samples. The rate of isolation of *S. aureus* was not found to be significantly influenced by the age, gender or the health status of the children.

Bacteria start to colonize the skin, respiratory tract, and intestines as soon as the newborn has left the sterile womb. The gut is sterile before birth but within 5 to 10 minutes after birth it is rapidly invaded by bacteria[12]. *Escherichia coli* and *enterococci* are present in infant stools within a few days and anaerobic bacteria of *Bifidobacterium, Clostridium* and *Bacteroides* genera within one week[3,4]. Other anaerobes are successively established, resulting in a highly diverse microflora as the child grows[13].

The study was restricted to children aged three and below because previous reports have indicated that a relatively permanent microflora is established in the human gut at two to three years of age[14]. This intestinal microflora, once formed, is reported to be specific to the host and resists alterations and modifications over time[4,13]. The choice of this age range was therefore to help in monitoring the dynamics of *S. aureus* colonization of the intestinal tract until the development of 'colonization resistance'[14] when the indigenous intestinal microbiota provides protection against colonization of the gastrointestinal tract by exogenous microorganisms[4,14].

The methods used in the isolation of the organisms used in the establishment of the rate of the intestinal carriage of *S. aureus* include the direct culturing of stools, rectal swabs and anal swabs[15,16]. In addition, the swabs from the perianal area (including the perineum and the groin or inguinal region) are generally accepted to define intestinal carriage[16, 17]. In the present study, stool culture was used in the estimation of intestinal carriage of *S. aureus*.

In studies reported as early as 1950s and 1960s, very high frequencies (ranging from 50 to 100%) for intestinal *S. aureus* carriage were reported in infants of ages ranging from 1 day to 12 months[18]. Much more recent studies showed that 64% to 75% of Swedish infants have *S. aureus* in their stools[19]. Hence, the carriage rate of *S. aureus* by the children in the present study is lower.

In most of the reported studies the rate of colonization by *S. aureus* seemed to increase from the age of 1 day to an average maximal value at the age of 1 month to 6 months[19]. The result in respect of the age of carriage agreed with the reports of these workers in that, in this study also, it was observed that the rate of isolation of *S. aureus* increased from 16.2% at age 0 to 15 days reaching the maximal value of 27.3% at about 1 month of age then decreasing steadily to 10.5% at about 18 to 24 months. *S. aureus* was not isolated from any of the children above the age of 2 years.

The variation in the carriage rate obtained in the studies mentioned above vis a vis the rate obtained in this study is not unexpected since literature has shown that a wide variety of factors may influence the process of colonization of the human gut with bacteria species[209]. Factors like delivery and feeding mode, social contacts and the degree of environmental hygiene were noted to play very important roles in these cases. Infants feeding pattern and environmental exposure to bacteria have also been reported to be of major importance[14]. Thus, considerable variation may occur in the intestinal carriage pattern in infants from one environment to another.

S. aureus strains isolated from the feacal samples of children were assayed for susceptibility to a range of antibiotics some of them being frequently administered in this environment. An assessment of the resistance patterns of intestinal *S. aureus* strains may give an insight into the ecological consequences for these organisms of antibiotic treatment of individuals and antibiotic usage in the society at large. The result showed that the *S. aureus* isolates were resistant to many of the antibiotics against which they were tested.

In this study, 37(90.2%) of *S. aureus* were resistant to penicillin. The rate of penicillin resistance in this study was comparable with that obtained by other workers who had worked on stool samples

of children[21,22]. In addition, comparable rates of resistance had been reported in *S. aureus* isolated from healthy students and hospital patients in the study environment[23].

In this study, 39.0% of the *S. aureus* isolates were found to be oxacillin resistant. This result, while it was not unexpected in view of the high rate of resistance to antibiotics in this environment, is actually on a very high side with the observation that none of the *S. aureus* isolates obtained from the faecal samples of children in Spain and Sweden was reported to be resistant to oxacillin[14,22]. However, one study reported an oxacillin resistance rate of 70% in *S. aureus* isolated from the faecal samples of children in Poland suggesting the laxity of antibiotic control in that country[24].

Despite this situation however, one interesting report was published that documented carriage of MRSA in children from foreign countries adopted by Swedish families[25]. The intestinal MRSA carriage rate of these children was 48% and is comparable to the 39% obtained in this study. This suggests that MRSA prevalence is an index of socio-political development in that controls are almost invariably absent in those countries where there is poor infrastructure and other development. It also suggests that occurrence of high level resistance in a particular section of the world could no longer be localised in the face of the current rate of international travels. This observation was also made by other workers who also reported the dissemination of virulent clones of MRSA (Clone H) from children transferred from West Africa to Switzerland for surgical operation[26].

The importance of the isolation of MRSA from the faecal samples of children in this study is also seen in the fact that the role of intestinal *S. aureus* as a causative agent for enteritis or antibiotic associated diarrhoea (AAD) and as a risk factor for other infections gained renewed interest with the spreading of MRSA. MRSA has been documented as a cause of AAD in hospitalized patients[5]. Although the result of this study did not show any relationship between isolation of MRSA and occurrence of diarrhoea among the children, the observations of these workers suggest the possibility of a cause and effect relationship.

Results show that all the isolates were sensitive to gentamicin with low resistance rate to clindamyin (7.3%), ciprofloxacin (12.2%), amikacin (14.6%), tobramicin (14.6%) and fusidic acid (22.0%). The fluoroquinolones are not recommended for therapy in children, therefore the remaining antibiotics could serve as therapeutic options for infections by multidrug resistant strains of this organism in children of these age groups.

It is important to educate patients and health care workers on strategies to limit further spread of infections due to *S. aureus* such as skin and soft tissue infection. For example, loose stools in hospital settings from children with faecal *S. aureus* can be a source of infections. There should be proper environmental cleaning and decontamination of equipments. Hospital policies should include the importance of training in infection control.

Conclusion

The study suggests that children in Ile-Ife harbor multiply antibiotic resistant *S. aureus* strains in their intestine. This suggests the need for appropriate antimicrobial use to halt or at least limit the spread of resistance. The prevention of or therapy for intestinal carriage of *S. aureus* should also be clinically beneficial. Gentamicin should be the first line drug for treating infections caused by these organisms in these age groups. This problem demands constant monitoring.

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