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BACTERIA COLONIZATION OF THE NASOPHARYNX OF STUDENTS IN A TERTIARY INSTITUTION SETTING

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ABSTRACT

Background: The nasopharynx harbours various diverse microflora in children and can be complex ecosystem. Most of these flora are commensals in adults which are not often investigated. The study analysed such diversity among apparently healthy students, the antibiotic susceptibility and products of resistance between September 2015- February 2016. **Methods:** Two hundred (200) apparently healthy students aged 16-30 years were recruited for the study. Each sample was collected from participant's pharynx using a sterile cotton-tipped applicator that was initially dipped in sterile normal saline and introduced into sterile thioglycollate fluid media. All such samples were incubated at 37^{o}C aerobically for 24 hours for growth. Duplicate samples were prepared for anaerobes and incubated with 5% CO_2 in anaerobic jars and further studied. Antibiotic susceptibility tests were carried out by the Kirby Bauer disc diffusion method using the Mueller Hinton as plating agar. Molecular characterization of resistant and virulence genes was carried out by polymerase chain reaction (PCR). **Results:** The study revealed 39% of the study population aged 19-21 years, had the highest carriage rate. Pathogenic *S. aureus* constituted 52(33.55%), streptococci 7(4.52%). 79 predominant pathogens were recovered with high resistance to the beta lactams, 70(88.62%) compared to 9(11.39%) to ciprofloxacin and 11(13.92%) to gentamycin. Of the 12 *S. aureus* isolates amplified and electrophoresed, 9 isolates presented the *nuc* gene, three isolates carried the aac-3-iv resistant gene while7 isolates carried the *mecA* resistant gene. However, none of the isolates carried the *Luk-pv* gene. **Conclusion:** The study revealed the extent to which the beta lactams antibiotics 88.62% were ineffective compared to 13.92% gentamycin and 11.39% ciprofloxacin.

KEYWORDS: Nasopharynx, pathogenic bacteria, antibiotics and resistance.

INTRODUCTION

The nasopharynx is known to display various types of microbial flora thus making it a complex ecosystem.[1] While such display occurs among children early in life,[2] its significance is less in the elderly. Colonization of the nasopharynx and the respiratory tract in humans by indigenous flora is competitive and often eliminate potential pathogens by reducing their number through microbial antagonism a succinct aspect of innate immunity.[3] Being the first line of host defence against invading pathogens, innate immunity is spontaneous and non-discriminatory. Innate response manifests through phagocytes such as macrophages and dendritic cells which trigger the immune system as well as humoral factors and surface barriers.[4] Among the families of bacteria of the nasopharynx are Moraxellaeceae, Streptococcaceae Corynebacteriaceae, Pasteurellaceae and Staphylococceae.[5] *Moraxella catarrhalis* and *Staphylococcus aureus* are often transient colonizers of this complex microbiota that can also cause diseases.[6]

It has been shown that many factors influence nasopharyngeal carriage rate among children and adults.[7, 8, 9] We are not aware of any study on bacterial carriage rate in the nasopharynx of adults in this environment hence our determination to undertake this study among apparently healthy students in a tertiary institution setting. We believe that the data obtained from our study will elucidate information of the dynamics of pathogens flux in adults nasopharynx that will contribute to knowledge in this area and thereby assist clinicians with their data base.

METHODS

Study population and design

This was a prospective study during which students' participation was based on physical appearance. A total of 200 apparently healthy students aged 16-30 years in their freshmen, sophomore, junior and senior years respectively were recruited. Participation was voluntary and each participant was administered structured questionnaire for the study.

Sample collection, isolation and identification

Each sample was obtained from each participant's pharynx using a sterile cotton-tipped applicator (Evepon, Industrial Limited, Onitsha, Anambra State, Nigeria) that was initially dipped in sterile normal saline and introduced into sterile thioglycollate fluid media. All such

samples were incubated at 37^{o}C aerobically for 24 hours for growth. Duplicate samples were prepared for anaerobes and incubated with 5% CO_2 in anaerobic jars (AnaeroPack Jar 2.5 Liter, Order No. 50-25, product of Mitsubishi Gas Chemical Company Co., Inc., 5-2 Marunouchi 2-chome, Chiyoda, Tokyo, Japan) for 48hrs. For biochemical characteristics and identification, a loopful of each culture 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^{o} C for 48 hr. Only plates on which colonies appeared were examined. Each distinct colony appearing on agar plates was picked and further studied. Identification was based on cultural, morphologic characteristics such as size, elevation, opacity and colour on media plates. Colonies from each culture medium were Gram stained and processed for biochemical identification using the Analytical Profile Index (API) 20E and API Staph (Biomerieux, France). Sensitivity to Taxo A disc (0.04 units of bacitracin) and Taxo P disc (5 µg) ethylhydrocupreine hydrochloride (optochin; BD Diagnostics, Difco Laboratories, Detriot, Michigan, USA) were also employed for identification for Streptococci. Antibiotic susceptibility tests were carried out by the Kirby Bauer disc diffusion method using the Mueller Hinton as plating agar (Oxoid Ltd, Basingstoke, Hampshire, England). The antibiotics included erythromycin (15 µg), gentamicin (10 µg), agumentin (30 µg), tetracycline (10 µg), nitrofurantoin (200 µg), ciprofloxacin (5 µg), ceftriaxone (30 µg), oxacillin (1 µg), Ceftazidime (30 µg) and cefuroxime (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 colony in 200 µl of sterile distilled water in labelled eppendorf tube and centrifuged at 13,000rpm for 3mins. The suspension was heated at 100^{0}C for 10 minutes in a microwave machine (Haier thermocool), cold shocked in ice for 2 mins and vortexed (Gallenkamp spin-mix, Germany). The suspension was re-centrifuged at 10,000 rpm for 1 min and then stored at -20^{o}C for DNA amplification.[10]

PCR detection of *S. aureus nuc, mecA, aac-3-iv* and *pvl* genes

Multiple antibiotic resistant *S. aureus* are known to carry resistance genes as well as the virulence genes.[11] The study also determine resistance genes such as mecA genes which codes for methicillin resistance and aac-3-iv which also codes for gentamicin resistance. Details of specific 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 are given in Table 4.

A 25 µl mixture containing 4 µl of 10X buffer, 0.5 µl $MgCl_2$, 3 µl dNTPs, 0.2 µl Taq polymerase, 1 µl of the forward primer, 1 µl of the reverse primer and 5µl of extracted DNA was prepared in a PCR vial. The vial was placed in a programmed thermocycler (iCycler; Bio-Rad, Milan, Italy) for optimum conditions with an initial denaturation at 94^{o}C for 5 minutes, followed by 30 cycles of denaturation at 94^{o} C for 1 minute, annealing at 55^{o} C for 1 minute and extension at 72^{o}C for 1 minute. A final extension procedure was carried out at 72^{o}C for 10 minutes.

Agarose gel electrophoresis

Amplified PCR products were electrophoresed on 1% agarose gel (1 g of agarose powder-Promega, Madison, USA) 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.

Statistical Analysis of Data

Statistical evaluation was done using student *t*- test and one- way analysis of variance (ANOVA) with $P \leq 0.05$ as the indicator of statistical significance. SPSS (Chicago, IL, SPSS Inc., 2007) version 17.0 for Windows® was used to perform the analyses.

RESULTS

Table 1 showed a total of 200 students participated in the study, aged 16-30 years. Between 16-18 years, 7 (3.5 %) students participants numbering 3 males and 4 females followed by 78 (39%) aged 19-21 years, 28 males and 50 females while 63(31%) aged 22-24 years, 31 males

and 32 females. Furthermore, 37(18.5%) participants aged 25-27 years, 17 males and 20 females while 15(7.5%), age range 28-30 years, 6 males and 9 females Table 1.

The profile of bacterial isolates recovered from the nasopharynx of the study participant is shown in table 2. The results showed 312 bacterial isolates were cultured from the nasopharynx of 200 participants averaging 1.56 bacterial isolate per participant, consisting of 155(49.67%) gram positive cocci, made up of pathogenic *S. aureus* 52(33.55%), coagulase negative staphylococci 79(50.96%), enterococci 1(0.65%), streptococci 7(4.52%) and micrococci 16(10.32%) each. While the gram positive rods cultured were 119(38.14%), 6(5.04%) were spore formers and 113(94.95%) were non spore formers. Furthermore, the results also showed 38(12.18%) gram negative bacterial isolates were cultured from the nasopharynx of the participants, 20(52.6%) were lactose fermenters and 18(47.4%) non lactose fermenters Table 2.

Table 3 revealed the profile of antibiotic sensitivity pattern of selected predominant pathogens recovered from the nasopharynx of the students. The results showed, 79 predominant pathogens were tested to 10 antibiotics belonging to 7 different classes. Among the beta lactams, 31(39.25%) of the isolates were resistant to oxacillin and 39(49.37%) to augmentin. However, among the cephalosporins, 38(48.10%) of the isolates were resistant to ceftriaxone, 58(73.41%) to ceftazidime and 49(62.02%) to cefuroxime. Furthermore, among the macrolides, 54(68.35%) of the isolates were resistant to erythromycin, while 29(36.70%) were resistant to tetracycline, 9(11.39%) to ciprofloxacin, 32(40.5%) to nitrofurantoin and 11(13.92%) to gentamycin Table 3.

Table 4 showed the primers used to amplify sequences of the resistance and virulence genes, 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.

Table 1: Profile of age distribution among participants

Age range (yrs)	Total No (%)	Males (No %)	Females (No %)
16-18	7(3.5)	3(42.9)	4(57.14)
19-21	78(39)	28(35.89)	50(64.10)
22-24	63(31.5)	31(49.20)	32(50.80)
25-27	37(18.5)	17(45.94)	20(54.05)
28-30	15(7.5)	6(40)	9(60)
Total	**200**	**85**	**115**

Table 2: Profile of bacterial isolates cultured from the nasopharynx of participants

Bacterial Isolates	Total No (%)	Males No (%)	Females No (%)	P values
Gram positive cocci				
Staphylococcus aureus	52	23(44.23)	29(55.76)	
Coagulase negative staphylococci (CONs)	79	36(45.6)	43(54.4)	
Enterococci				
Enterococcus spp.	1	0(0.00)	1(100)	
Streptococci				
Streptococcus pneumoniae	7	3(42.85)	4(57.14)	
Micrococci				
Micrococcus luteus	14	4(28.60)	10(71.40)	
Micrococcus haemolyticus	2	2(100)	0(0.00)	P=0.10
Gram positive rods (Non spore formers)				
Corynebacterium xerosis	36	24(66.7)	12(33.33)	
Corynebacterium ulcerans	29	19(65.52)	10(34.48)	
Corynebacterium diphtheriae	7	3(42.86)	4(57.14)	
Corynebacterium amycolatum	4	0(0.00)	4(100)	
Corynebacterium urelyticum	4	1(25)	3(75)	
Corynebacterium riegelii	4	3(75)	1(25)	
Corynebacterium jeikieum	3	2(66.7)	1(33.3)	
Arcanobacterium haemolyticum	6	4(66.7)	2(33.3)	
Lactobacillus spp	17	8(47.1)	9(52.9)	
Listeria monocytogenes	3	1(33.3)	2(66.7)	
Bacilli (Spore formers)				
Bacillus subtilis	3	2(66.7)	1(33.3)	
Bacillus cereus	3	(0.00)	3(100)	P=0.01
Gram Negative rods (Lactose fermenters)				
Klebsiella pneumoniae	14	6(42.9)	8(57.14)	
Citrobacter freundii	4	3(75)	1(25)	
Escherichia coli	2	1(50)	1(50)	
Non lactose fermenters				
Pseudomonas aeruginosa	9	2(22.2)	7(77.8)	
Pseudomonas fluorescens	6	1(16.7)	5(83.3)	
Bordetella pertussis	2	0(0.00)	2(100)	
Clostridium difficile	1	1(100)	0(0.00)	P=0.02
Total	312	149	163	

Table 3: Profile of multiple antibiotics resistance index of predominant pathogens cultured from the nasopharynx of participants.

Classes of antibiotics	Antibiotics tested	Total No of bacterial isolates tested	No(%) resistant	No(%) susceptible	Multiple Antibiotics Resistance (MAR) Index
β- lactams	Oxacillin	79	31(39.25)	48(60.75)	0.39
	Augmentin	79	39(49.37)	40(50.63)	0.49
Cephalosporins	Ceftriazone	79	38(48.10)	41(51.89)	0.48
	Ceftazidime	79	58(73.41)	21(26.58)	0.73
	Cefuroxime	79	49(62.02)	30(37.97)	0.62
Macrolide	Erythromycin	79	54(68.35)	25(31.65)	0.68
Tetracycline	Tetracycline	79	29(36.71)	50(63.29)	0.37
Fluoroquinolones	Ciprofloxacin	79	9(11.39)	70(88.60)	0.11
Nitrofurans	Nitrofurantoin	79	32(40.5)	47(59.49)	0.40
Aminoglycosides	Gentamycin	79	11(13.92)	68(86.07)	0.14

Table 4: Profile of primers used for the detection of resistance and virulence genes.

Target genes	Oligonucleotide sequence 5'-3'	Size of target region (bp)	Annealing temperature (^{o}C)	References
nuc	F:GCGATTGATGGTGATACGGTT R:AGCCAAGCCTTGACGAACTAAAGC	276	55	[20]
mecA	F:GTAGAAATGACTGAACGTCCGATAA R:CCAATTCCACATTGTTTCGGTCTAA	310	57	[21]
aac-3-iv	F:AGTTGACCCAGGGCTGTCGC R:GTGTGCTGCTGGTCCACAGC	286	55	[22]
Luk-pv	F:ATCATTAGGTAAAATGTCTGGACATGATCCA R:CATCAAGTGTATTGGATAGCAAAAGC	433	57	[21]

Legend: F=forward primer, R=reverse primer

Table 5: Characterization of *Staphylococcus aureus* cultured from the nasopharynx of participants.

Isolate code	*Nuc* gene	*aac-3-iv* gene	*mecA* gene	*Luk -pv* gene
ANA160	+	+	+	-
BLD68A	+	+	+	-
BLD38A	+	+	+	-
BLD31A	-	-	-	-
BLD188A	+	-	+	-
BLD184B	+	-	+	-
BLD37A	+	-	+	-
BLD18A	-	-	-	-
BLD73B	+	-	-	-
BLD134	+	-	-	-
BLD181A	-	-	+	-
BLD129	+	-	-	-

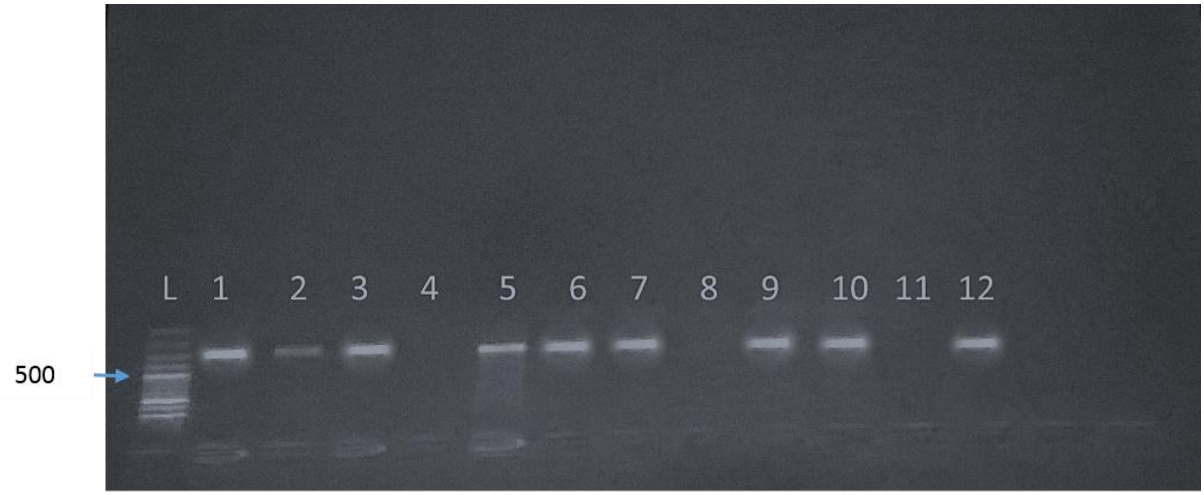

Figure 1: PCR detection of *nuc* gene in *Staphlococcus aureus* isolates, (*nuc* gene amplicon size 276 bp, L- 100 bp size marker, Lanes 1-12- *S. aureus* isolates).

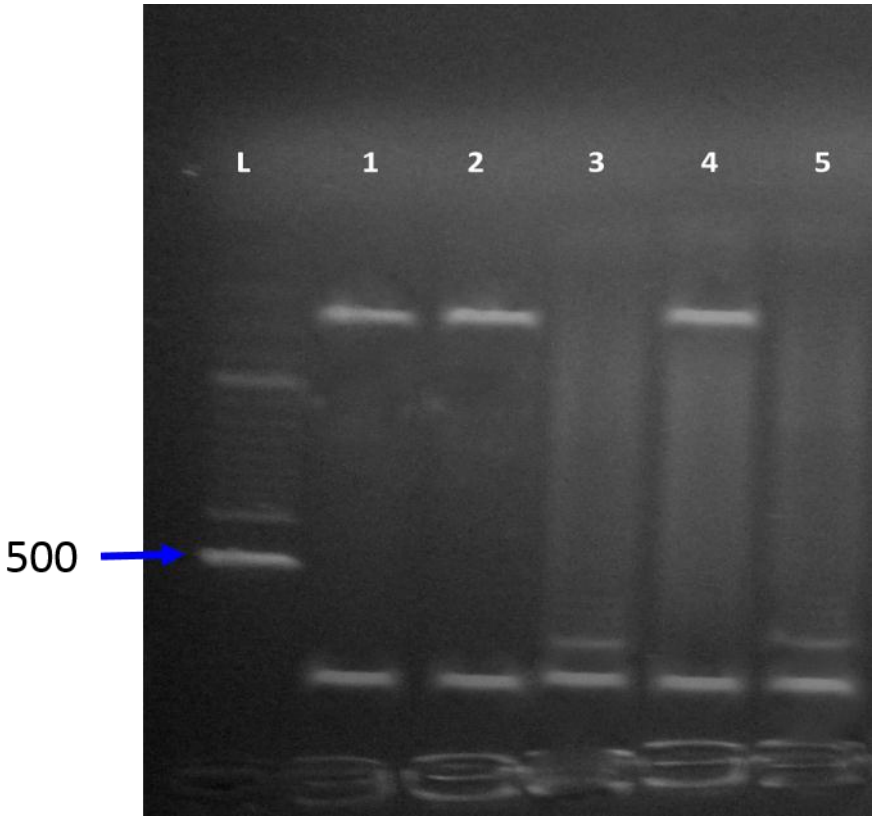

Figure 2: Agarose gel electrophoresis of PCR product for detection of *aac-3-iv* gene (amplicon size 286 bp, L- 100 bp size marker).

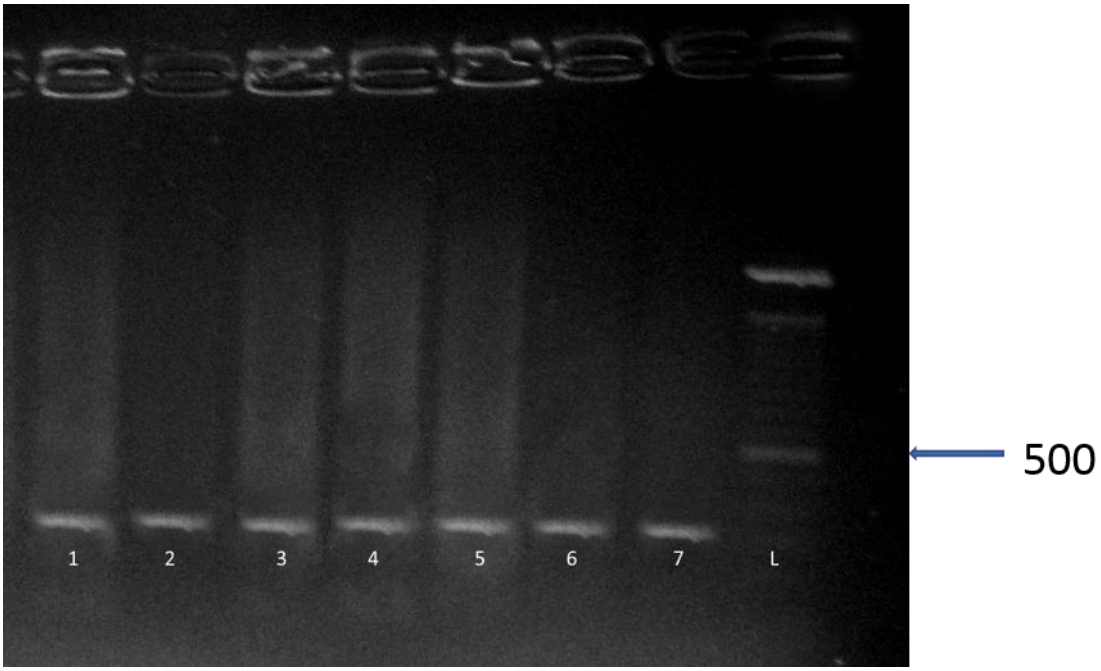

Figure 3: Agarose gel electrophoresis of PCR product for detection of *mecA* gene (amplicon size 310 bp, L- 100 bp size marker).

DISCUSSION

The study determined the prevalence of pathogenic bacteria that colonized the nasopharynx of apparently healthy adults in a tertiary institution setting, the antibiotic susceptibility profile and genes for resistance to antimicrobials. The result showed the participants aged between 19-21 years had the highest carrier rate 78/200=39%, followed by 22-24 years 63/200=31.5%, and 25-27 years 37/200=18.5%. Table 1.

Our result also revealed, that among the 200 participants, 312 bacterial isolates were recovered, of which pathogens represented 86(26.83%) of the total bacterial isolates recovered. Among these pathogens *S. aureus* was the predominant isolate seen 52/86=60.46%, followed by *Klebsiella pneumoniae*14/86=16.27%. In addition, *Streptococcus pneumoniae* and *Corynebacterium diphtheriae* represented 7/86=8.14% carriage rate Table 2. Studies have shown that among the families of bacterial associated with nasopharynx[5], *Moraxella catarrhalis* and *Staphylococcus aureus* are often transient colonizers of this complex microbiota that are etiologic agents of diseases.[6] Carriage rates of bacterial associated with the nasopharynx is a function of innate and adaptive immunity. Innate response expresses a set of germ-line encoded pattern recognition receptors (PRRs) which recognize many different pathogens associated molecular patterns (PAMPs).[12] Among these PAMPs are : lipopolysaccharides (LPS), peptidoglycan(PGN), lipoteichioc acid (LTA)

lipoarabinomannan (LAM), lipopeptides of bacterial cell wall, yeast wall mannans[13] bacterial DNA and Flagellin that are capable of stimulating innate immunity responses.[14] The recognition of PAMPs and PRRs has been shown by investigators to trigger phagocytosis and /or activate pro-inflammation pathways.[15] The dominance of *S. aureus* among the participants may not be unusual because nasal carriers of the organism has been reported between 8-30% in the last 35 years in Ile-Ife in this environment.[16]

Therefore full analysis of the *S. aureus* isolates were carried out to understand the existence of resistance genes. The results also showed that of the 12 isolates amplified and electrophoresed, 9 isolates presented the *nuc* gene, three isolates carried the aac-3-iv resistant gene while7 isolates carried the *mecA* resistant gene. However, none of the isolates carried the *Luk-pv* gene. (Table 5 and Figures 1-3).

The study revealed the ineffectiveness of the beta lactams antibiotics compared to gentamycin and ciprofloxacin Table 3.While external factors such as vaccines and antibiotics treatment has also been shown to contribute to pathogens of the nasopharynx, they also often interact with one another to produce positive and negative flux constantly experienced in the host.[17] In addition, factors which differ in children and adults such as age, seasonal changes, the type of child day care attended, number of siblings in a family, acute respiratory illness and sleeping position) in children can influence carriage rates. Similarly among adults conditions such as chronic obstructive pulmonary disease (COPD), obesity, immunosuppression and allergy can also stimulate carriage rate.[18]

Interestingly, Corynebacteria were commensals recovered as predominant organisms in this study corroborating previous studies[19] which may have reduced the carriage rate.

CONCLUSION

The study showed the beta lactam antibiotics were ineffective at least *in vitro* tests as 79/312=26% of the pathogenic isolates recovered from the nasopharynx of the participants were multiply resistant. The high resistance of bacterial isolates recorded among apparently healthy students is worrisome and portends some alarm to the community. The existence of multi-resistance bacterial isolates suggests caution primarily to the community in the event of an epidemic in the environment which is epidemiologic importance for the control of diseases.

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