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Antibiotic resistance pattern of methicillin-resistant *Staphylococcus aureus* isolated from children under the age of five Years in Anambra State.

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Abstract

Neonates are exposed to *Staphylococcus aureus* shortly after birth and can become colonized quickly after contact with adult skin or the environment. Methicillin-resistant *S. aureus* (MRSA) constitutes part of the growing global health problem associated with an increasing number of infections and often multidrug resistant in nature which now poses serious therapeutic problems to clinicians. Eighty-three (83) samples were collected which were cultured on appropriate bacteriological media. Bacterial isolates (*S. aureus*) were identified by standard biochemical tests and confirmed using PCR targeting the 16S rRNA. The MRSA was determined using Oxacillin antibiotic disk and confirmed by the presence of *MecA* gene. Antibiotic susceptibility of the MRSA isolates to eleven antibiotics was performed according to Clinical Laboratory Standard testing Institute (CLSI) guidelines. Out of the 83 individuals tested, 25 yielded *S. aureus* of which 22 (88.0%) of them were MRSA positive, 10 (45.5%) females and 12 (54.5%) males. The antibiotic resistant pattern of the 22 MRSA isolates showed Quinupristin/Dalfopristin 13 (59.1%), Fusidic acid 6 (27.3%), Linezolid 8 (36.4%), Clindamycin 10 (45.5%), Vancomycin 5(22.7%), Cefepime 3(13.6%), Doxycycline 4(18.2%), Sulphamethoxazole 22(100%), Fosfomycin 7(31.8%), Cephalexin 7(31.8%), Trimethoprim 19(86.4%). The MIC determination for vancomycin from MRSA isolates showed antibiotic concentration of 4.9 µg/ml and 8.7 µg/ml. This work showed that there is high prevalence rate of resistance to many classes of antibiotic warranting continued surveillance and antimicrobial stewardship. Therefore effective antibiotic susceptibility test should be conducted before prescribing an antibiotic to patients, in as much as patients should strictly adhere to antibiotic prescription to mitigate abuse of drugs.

Keywords: Methicillin-resistance, antibiotics, prevalence, S. aureus, susceptibility.

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Introduction

Staphylococcus aureus is one of the major human pathogens, significantly contributing to Hospital and community acquired infection (Ugwu et al., 2016). The emergence of Methicillin-Resistant *Staphylococcus aureus* (MRSA) that is cross resistant to most beta-lactams reduces the treatment options for staphylococcal infections (Saravanan et al., 2013).

Methicillin-resistant *Staphylococcus aureus* (MRSA) strains have been reported by many investigators to have developed resistance to antibiotics through enzymatic degradation, alteration of bacteria proteins, efflux of antibiotics

and the risk factors include childcare/crowded living conditions and indiscriminate use of antibiotics (Samore et al., 2001; Tenover, 2006). Presence of resistance plasmids (R-plasmids) in cells makes the cells resist the effect of antibiotics and makes chemotherapy of infections very difficult. Plasmids are extrachromosomal genetic elements that are transferred from one cell to another through horizontal gene transfer (Sheikh et al., 2003).

In neonates and children, the prolonged hospitalization, and antibiotic exposure, has been indicated to increase risk of infection with multi-resistant pathogens, the greater the duration of exposure, the greater the risk of the development of resistance irrespective of the severity of the need for antibiotics (Shane and Stoll, 2014).

MRSA infections has been reported to be responsible for more deaths in the US each year than AIDS according to Kavanagh et al. (2017). MRSA-related deaths was also of major concern to England and Wales (Klevens et al., 2007) as MRSA is seen to have increased the rate of mortality in UK since 1993 (Blot et al., 2002). Most African countries including Nigeria (29.6%), Kenya (27.7%), Cameroon (21.3%), Cote D'Ivoire (16.8%), and Morocco (14.4%) (Kesah, 2003) have recorded higher isolation rate of MRSA. However, there are great chances of MRSA to be transmitted outside hospital for the fact that there is no checkup for MRSA decolonization when patients are discharged from hospitals and this raises serious concerns about the possibility of transmission of MRSA outside the health care system. The possibility of MRSA becoming the common form of *S. aureus* in a community will make treatment much more difficult (Akujobi et al., 2013).

As resistance towards antibiotic becomes more common, a greater need for alternative treatments arises. However, despite a push for new antibiotic therapies, there has been a continued decline in the number of newly approved drugs. Antibiotic resistance therefore poses a significant problem (Emeka-Nwabunnia et al., 2015). Development of new antimicrobials presently is difficult, coupled with increasingly common and novel-resistance mechanisms by organisms which provide challenges for clinicians. For this reason, though clinicians and scientists, anticipate for the development of new antimicrobial agents, are looking forward to investigate possibilities of using previously discarded agents or improve established agents in a different way to ameliorate the situation (Benjamin, 2014). In one of such ways like testing for the different concentrations of such antibiotics that organism have developed resistance for and determining the new minimum inhibitory concentration (MIC) for which the resistant strain of the organism will be susceptible to.

This necessitates the need for many epidemiological investigations which have been focused on *S. aureus* strains isolated from patient's specimens or invasive infections, but only a few have studied the prevalence of MRSA collected from pediatric units in Anambra state.

Methodology

Study Area and Population

The study was carried out in Anambra State located in the South Eastern part of Nigeria. The area was selected because of some suspected risk factors for MRSA infection and colonization such like over crowdedness, poor sanitation and hygiene and also due to the familiarity of the area by the researcher. The study was conducted on 83 children under the age of five (0 month to 59 months) presenting at the Children Out-patient clinic (CHOP), paediatric wards and children emergency ward of the two selected hospitals; De Vince Memorial Children Specialist Hospital Ugwuagba, Obosi and General Hospital Onitsha in Anambra State.

Ethical Considerations

Written ethical approval was obtained from the State Hospital Management Board (SHMB) (Ref No. SHMB/AD/488/Vol II/44) under the jurisdiction of the Ministry of Health Anambra State (Ref No. MH/AD/12/T/III/313). Signed informed consent was also obtained from participant's care-givers included in the study.

Identification of bacterial Isolates

Samples were collected from nostrils, ear, skin and wound from children using sterile swab sticks while sterile needles was used to collect blood samples. The swabs were immediately inoculated on Baird-Parker media (Titan Biotech LTD, India. Lot no. M3F8HP01) and incubated for 24 h. All Gram-positive cocci isolates that were in clusters were subjected to

standard biochemical characterization tests; coagulase and catalase test for *Staphylococcus aureus* (Cheesbrough, 2006). The isolates were confirmed by molecular method. The chromosomal DNA was extracted by boiling according to the method of Zhang et al. (2004), by sub culturing in 5 mL of Mueller Hinton broth and incubated at 37 ºC overnight preceding DNA harvesting. Molecular identity of the cultures was tested based on PCR targeting the 16S rRNA according to McClure et al. (2006). Crude bacterial lysates of all cultures were prepared targeting a 756-bp internal fragment of the gene with primer concentrations of 100 µM as follows; forward: 5'- AAC TCT GTT ATT AGG GAA GAA CA - 3', and reverse 5'- CCA CCT TCC TCC GGT TTG TCA CC -3' (Inqaba Biotechnical company Pty, South Africa). PCR reaction mixture was optimized by adding to each 25 µL eppendorf tubes, 16.4 µL of sterile distilled deionised water, 5 µL of PCR buffer, 1.5 µL of 1x $MgCl_2$, 0.5 µl of dNTP, 0.25 µL each of the forward and reverse primers and 0.1 µL of *Taq* DNA polymerase (Promega, USA). To completely make it a 25 µL reaction mixture, 1µL of DNA from the processed clinical isolates was added. PCR was carried out in a thermal cycler (Eppendoff Vapoprotect, Germany) with the reaction cycles consisting of an initial denaturation of 94 ºC for 5 min; 34 cycles of 55 ºC for 30 seconds, 52 ºC for 1min and 72 ºC for 1 min. A final extension step at 72 ºC was continued for another 10 min. The PCR products were resolved on 2% agarose gels containing 0.5 µg/mL ethidium bromide and visualised on UV transilluminator using a photo documentation system (Clinix Science, China).

Phenotypic detection of MRSA using the Oxacillin disc (Oxoid, England) and Molecular identification:

Oxacillin screening test was conducted on all the confirmed S. aureus isolates by the agar screening method. The *S. aureus* isolates were standardized to 0.5 M McFarland standards and were inoculated aseptically onto the Muller-Hinton agar plates. The plates were incubated for exactly 24 hours at 37^{0}C. Isolates positive to oxacillin disc were confirmed by the detection of the presence of *Mec* A gene**.** Polymerase chain reaction (PCR) assay according to the modified method of McClure et al. (2006), was used. Amplification of a single-target PCR bands corresponding to molecular size of 310bp for mecA gene with primers of MecA1 (5′ - GTA GAA ATG ACT GAA CGT CCG ATA A - 3′) and MecA2 (5′- CCA ATT CCA CAT TGT TTC GGT CTA A - 3′) that were easily recognizable in agarose gel stained with ethidium bromide was determined. The condition was optimized by assaying the two primers concentrations and other PCR components as follows: 2 µl of template DNA preparations was added to a 25 µl final reaction volume containing, 10.25 µl of distilled deionised water, 12.5 µl of master mix, 0.25 µl of *MecA* forward and backward primers, with thermocycling conditions set at denaturation at 94ºC for 5 mins, followed by 30 cycles of denaturation at 94ºC for 45 secs, annealing at 55ºC for 45 secs and extension at 72ºC for 75 secs and a final extension step at 72ºC for 10 mins. The PCR products were also resolved on 2% agarose gels containing 0.5 µg/mL ethidium bromide and documented using a gel documentation system (Clinix Science, China).

Antibiotic susceptibility testing (AST)

The antibiotic resistance pattern of the isolates was determined against eleven antibiotics using Kirby – Bauer disc – diffusion method following the CLSI (2014) guidelines. The inoculation of the confirmed Oxacillin-resistance *S. aureus* (MRSA) isolates was evenly distributed over the surface of the Muller-Hinton agar media in the petri dishes by rotating while streaking. The plates were allowed to set and the antibiotic sensitivity disc (Oxoid, England); Linezolid, Fusidic acid, Quinupristin/ Dalfopristin, Cephalexin, Clindamycin, Vancomycin Fosfomycin, Trimethoprim, Sulphamethoxazole, Cefepime and Doxycycline were aseptically placed on their surfaces using a sterile forcep. The plates were incubated at 37^{0}C for 24 h and the resultant Inhibition Zone Diameters (IZDs) measured and recorded. These were then interpreted as susceptible and resistant according to standard specifications of CLSI.

Minimum Inhibitory concentration of Vancomycin Resistance Staphylococcus aureus

Two methicillin-resistant *S. aureus* isolates which showed resistance to vancomycin antibiotics disc (30µg/mg) were further subjected to Minimum Inhibitory Concentration test. Varied concentration of the antibiotic was

prepared above the CLSI recommended Minimum inhibitory Concentration standard for Vancomycin which is 30µg/mg (CLSI, 2008). The MIC of the Vancomycin antibiotic was conducted using broth dilution method on the two isolated VRSA over the concentration ranges as follows; 3.0µg/ml, 3.3µg/ml, 3.7µg/ml, 4.9µg/ml and 8.7µg/ml. The vancomycin resistance was further confirmed using commercially prepared Brain Heart Infusion (BHI) agar plates (Remel©, USA) containing 6 µg/ml of vancomycin by inoculating 10 µl of 0.5 McFarland standard bacterial suspension on the plates.

Data collection for epidemiologic risk factors

Epidemiologic risk factors related to demographic and medical history of the study population (Age, sex, previous antibiotic history, and previous hospital exposure of self or family member, current symptoms and family size) were gathered using unstructured questionnaires. It was unstructured because it requires free responses in the respondents' words and style. It was designed to permit free responses from participants rather than limited to specific alternatives.

Statistical analysis

The data were analyzed using the One-sample t-test in SPSS version 20. Questionnaire data were recorded on the questionnaire forms and entered into a Microsoft Excel spreadsheet. Laboratory results were entered into the spreadsheet along with the corresponding participant's information as they become available. Descriptive statistics (including means, standard deviations, frequencies and percentage) were calculated for the socio-demographic variables. All tests were 2-tailed, and a $p<0.05$ was considered statistically significant.

Results

A total of 83 individuals participated in the survey of which 41 (49.4%) were males and 42 (50.6%) were females. The two sites of study and sample collected from each site were reported as 40 blood samples, 38 nose swabs, 3 wound swabs, 1 ear swab and skin swab Out of the 83 samples, 25 were found to be *Staphylococcus aureus*. All the 25 isolates amplified the expected 756 bp of the 16rRNA gene confirming the identity of the *S. aureus* (figure 2). Table 1 shows the clinical characteristics of patients with MRSA isolates.

Antibiotic susceptibility test result

Out of the 25 *S. aureus* isolates, 22 (88.0%) showed resistance to Oxacillin (Oxoid, England) indicating Methicillin-resistant *S. aureus*. The 22 isolates amplified the expected 310 bp of the *Mec A* gene confirming methicillin resistance (Figure 3). The antibiotic susceptibility profile showed that the 22 MRSA isolates were resistant to antibiotics tested in the following percentage, Cefepime (12%), Doxycycline (16%), Vancomycin (20%), Cephalexin (31.8%), Fosfomycin (18.2%), Fusidic acid (27.3), Linezolid (36.4%), Clindamycin (45.5%), Quinupristin/Dalfopristin (59.1%) and Trimethoprim (86.4%). MRSA isolates recorded complete resistance to Sulphamethoxazole (100%), as depicted by figure 1 below.

Table 1: Clinical characteristics of patients with MRSA isolates

Risk Factors	MRSA isolates No.(%)	p-value	95% C.I Lower
Antibiotic exposure			
Yes	14(63.6)	.000	0.42
No	8(36.4)		
Hospital Exposure			
Yes	11(50)	.000	0.27
No	11(50)		
Family member exposure* **			
Yes	5(22.7)	.021	0.04
No	17(77.3)		
Family size			
≥5	13(59.1)	.000	4.26
<5	9(40.9)		

*** Exposure to hospital environment

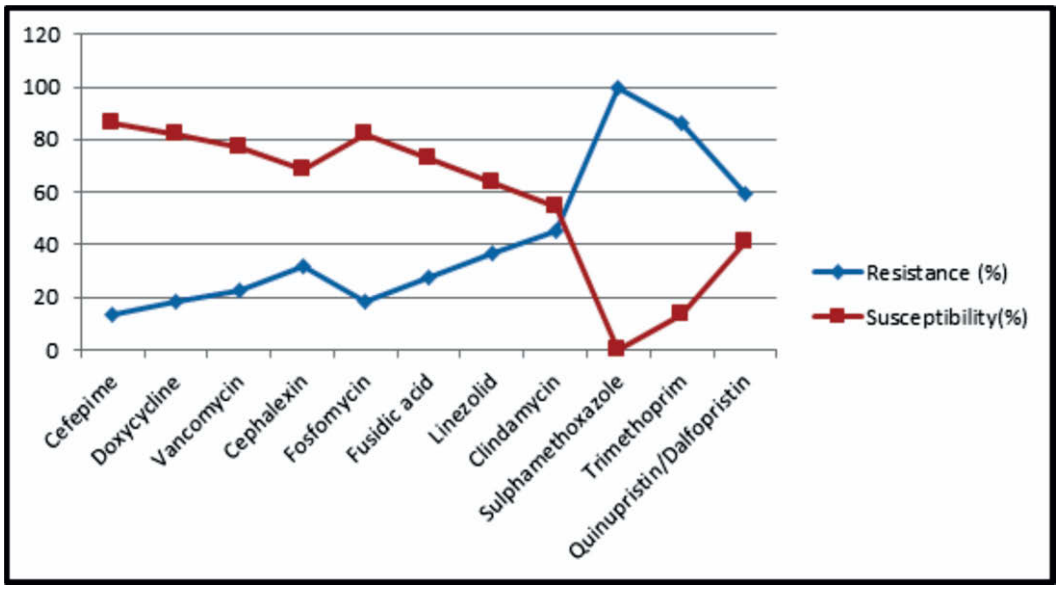

Figure 1: showing the susceptibility and resistance of the 22 MRSA isolates to different antibiotics

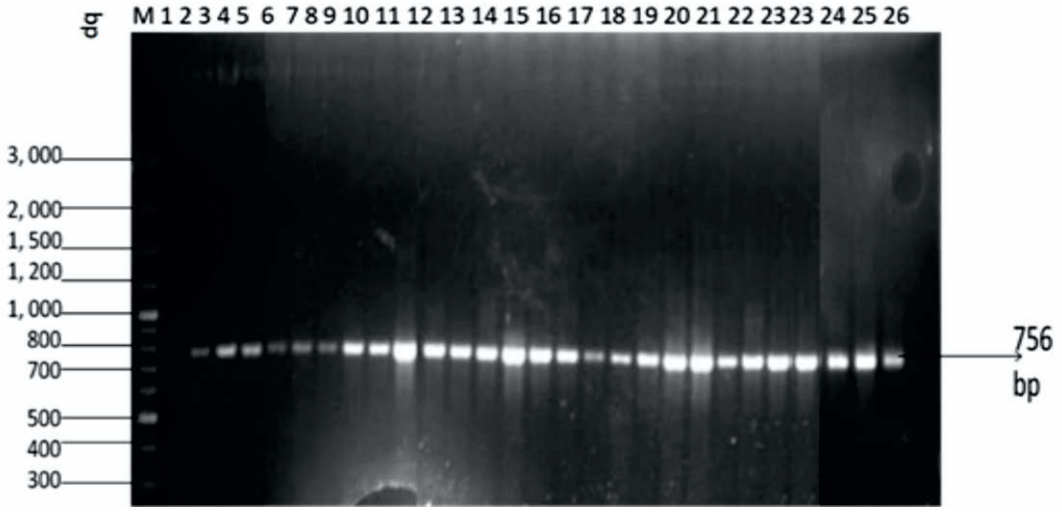

Figure 2: Showing the amplification of the targeted 16S rRNA confirming the organism to be S. aureus. Lane M represents the marker used, lane 1 and 2 represents negative and positive control respectively. Lane 3 - 26 represents the isolates.

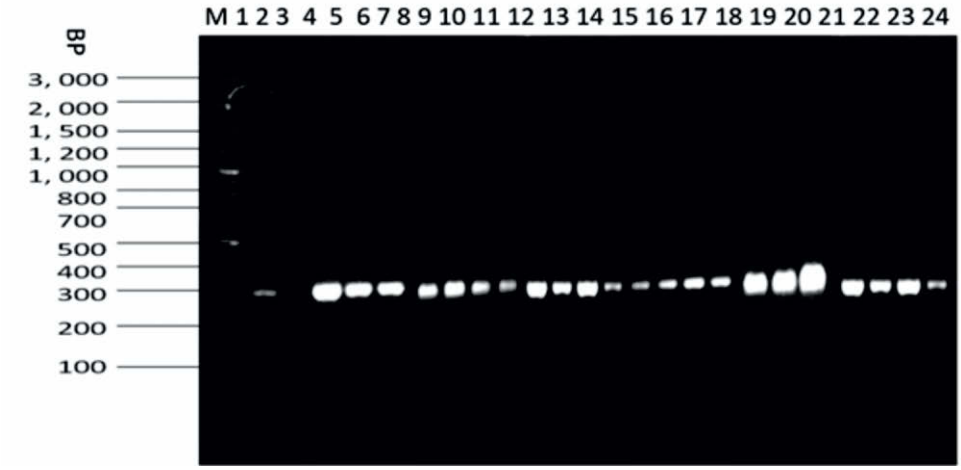

Figure 3: Showing the amplification of the targeted Mec A gene confirming the presence of methicillin-resistant gene. Lane M represents the marker used, lane 1 and 2 represents positive and negative control respectively. Lane 3 - 24 represents the isolates.

Discussions

From the results stated above patient colonization or infection with a resistant strains of MRSA is not random. Methicillin-resistant *S. aureus* (MRSA) were isolated from Patients were characterized by a greater risk of infection due to previous exposure to antimicrobial therapy and have recorded previous hospital exposure or had a close contact with someone who have been to such exposures, which have been reported by many research works such like previous work by Alrabiah et al., (2016) and Kejela and Bacha, (2013).

Out of 83 samples obtained, 25 samples showed positive *S. aureus* and of which 22 out of them were confirmed Methicillin-resistant, in this 17 out of the 22 MRSA isolates were obtained from nasal swab which showed high prevalence rate of nasal colonization among children. This is line with the report by Ugwu et al., (2016) who stated that nasal carriage of *S. aureus* represents an effective and progressively prevalent risk factor for subsequent *S. aureus* infection.

Among the 22 MRSA isolates from children included in the study, the statistically significant risk factors were obtained in children below the age of one year compared to other age range, children with previous antibiotic exposures and children whose family size is more than five. It was observed that patients under one year of age had the highest rate of colonization or infection with MRSA (12, 54.5%). This concurred with the previous research work by Alrabiah et al., (2016) and with the finding of study conducted by Gutierrez in California, USA, indicating that from 1985 to 2009, those less than one year of age had a higher colonization (Guteirrez et al., 2013). This further strengthens the evidence that young age is a risk factor for MRSA colonization and infection.

In this study, there is a significant association between children from whom MRSA were isolated that had previous hospital exposure and that of those that do not have previous hospital exposure ($p<0.05$), also with children with previous antibiotic history and those that do not have any previous antibiotic history ($p<0.05$). Since there was no significant difference between children with hospital exposures and those that do not have hospital exposure, or those that have contact with family member who have both hospital exposures and those that don't, then this can indicate the dissemination of the hospital acquired methicillin-resistant *S. aureus* (HA-MRSA) from hospital to the community (outside hospital) and such contributes to high prevalence of MRSA outside the hospital as reported by Kejela and Bacha (2013). This can be possible since there is no decolonization process done on patients before being discharge from the hospital and this increases the tendencies of distributing hospital acquired strain in the community. The possibility that these children must have acquired this MRSA outside the hospital is very high. Family member exposure had no significant association with the children having MRSA because most of the children especially those under one year of age are still tender and are mostly under the care of their mothers. Many reports have stated that over-crowdedness (McMichael, 2000; O'Malley et al., 2014), poor sanitation (Okeke, 2003) and antibiotic misuse associated with poverty (Chuc and Tomson, 1999; Kalu et al., 2008) can contribute to MRSA acquiring rate and all this are very common to the area of this study and this might have contributed to high prevalence of MRSA in this community. Furthermore, Nnachi et al., (2012) reported the high prevalence of MRSA among raw meat handlers in Onitsha, from this report cross-contamination can occur on the meat display table, even to the buyers and vice versa. This is of public health importance as it may play a potential role in transmitting the organism between animal and humans as well as the community. There was no observed significant difference in colonization rate between the male and female group. This observation is in line with the findings of Ajoke et al., (2012) in Jos, North Central Nigeria and Okwu et al., (2012) in Okada, Nigeria. They reported that sex is not a remarkable determinant in *S. aureus* colonization.

The antibiotic resistant profile showed that the 22 MRSA isolate showed high resistant to most of the antibiotics tested in this study as shown in the figures above. The resistance observed in most of the reserved antibiotics such as Linezolid, which is in the WHO list of essential medicine and most important in basic health system (WHO, 2014) is gradually increasing and this gradual increase in resistance to this antibiotic is a threat to the global health as the pipeline of antibiotic is becoming dry. Most of

the antibiotics are not usually found over the counter in the community pharmacy shop but since the MRSA isolates in this study showed resistance in virtually all the antibiotics tested this can be as a result of the injudicious use of broad spectrum antibiotics which this organism must have developed resistance to, this will constitute the mechanism of which they resist this set of antibiotics which are not easily found in community.

The Minimum Inhibitory Concentration (MIC) conducted on the methicillin-resistant vancomycin-resistant *S. aureus* inhibiting the growth of the organism which is above the CLSI recommended Standard showed that there is need to increase the concentration are required to treat MRSA which are resistant to vancomycin which is the drug of choice. This resistance is greater than that observed by Emeka-Nwabunnia et al. (2015) among *S. aureus* isolated from HIV patients. Believing that any time antibiotics are used, this puts biological pressure on bacteria that promotes the development of resistance (Hastings et al., 2004). Antibiotics should be used when necessary and needed to prevent or treat disease. Research has shown that most of the time, antibiotics are prescribed when they are not needed or they are misused by prescribing wrong dose to a patient. This not only fails to help patients; it might cause harm. Like every other drug, antibiotics have side effects and can also interact or interfere with the effects of other medicines. This inappropriate utilization of antibiotics unnecessarily advance antibiotic resistance (Kalu et al., 2008).

Antibiotics are a limited resource. The more that antibiotics are used today, the less likely they will still be effective in the future. Therefore, doctors and other health professionals around the world should be adopting the principles of responsible antibiotic use, often called antibiotic stewardship. Stewardship is a commitment to always use antibiotics only when they are necessary to treat, and in some cases prevent, disease; to choose the right antibiotics, and to administer them in the right way in every case. Effective stewardship ensures that every patient gets the maximum benefit from the antibiotics, avoids unnecessary harm from allergic reactions and side effects, and helps preserve the life-saving potential of these drugs for the future. Efforts to improve the responsible use of antibiotics have not only demonstrated these benefits but have also been shown to improve outcomes and save healthcare facilities money in pharmacy costs.

Conclusion

It has been confirmed that MRSA has become a major cause of illness and death in the society. This research has established that MRSA exists around us and children are most vulnerable. Educating the people on the risk factors associated with MRSA transmission and ways of combating its spread, avoiding drug abuse and self-medication should be encouraged as this will play a vital role in mitigating the increase in prevalence rate of infection and colonization.

Conflict of Interest:

The authors wish to declare that there is no conflict of interest.

Authors' Contributions:

Emeka-Nwabunnia, Ijeoma conceived the project. Ejigeme, Kenneth and Oguoma, Okechukwu carried out field collection and laboratory assays. I. Emeka-nwabunnia, carried out data analysis. All authors participated in manuscript drafting and revision.

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