# Multi-Drug Resistant Efflux Pumps among Clinical Isolates of *Staphylococcus aureus*

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**Abstract:** Against the background of high level antimicrobial resistance observed in isolates of *Staphylococcus aureus*, this study was conducted to determine the prevalence of multidrug resistant (MDR) efflux pumps among clinical isolates from University of Benin Teaching Hospital, Benin city. A total of 198 clinical isolates of *Staphylococcus aureus* obtained from various clinical specimens were used for the study. Disc susceptibility test, detection of MDR efflux pump among representative multidrug *Staphylococcus aureus* isolates as well as curing experiments on the positive efflux pump isolates were performed using standard techniques. The most active antibacterial agent was imipenem with a susceptibility profile of 32.32%. A total of 64(32.32%) of the 198 isolates of *Staphylococcus aureus* were MDR. Forty-seven (47) isolates of *Staphylococcus aureus* (23.74%) were recovered from urine samples. Compared to isolates from other specimens, the prevalence of MDR isolates was significantly higher (63.83%) in the urine specimens (P< 0.0001). The prevalence of MDR efflux pump was 9.09% (18/198) with a significant prevalence among isolates from urine (P=0.0032). All 18 *Staphylococcus aureus* isolates harbored resistant plasmids to the drugs that were used as substrates for efflux as well as to other drugs. Curing experiment revealed the loss of antibacterial resistance in some of the isolates after exposure to rifampicin. In conclusion, the isolates of *Staphylococcus aureus* used for this study were multidrug resistant with few plasmid-mediated; consequently had a multiple antibiotic resistant index (MARI) ≥ 0.2. Prudent use of antimicrobial agents is advocated to stem the tide of high bacterial resistance.

**Key words**: clinical isolates, efflux pump, multidrug resistant, *Staphylococcus aureus*

## INTRODUCTION

*Staphylococcus aureus* is considered to be a major pathogen causing infections ranging from skin and soft tissue infections, pneumonia, bloodstream infections, osteomyelitis, endocarditis, as well as toxin-mediated syndromes like toxic shock and food poisoning (Tong *et al*., 2015). Hospitalized individuals with decreased immunity and immunocompetent people are at a higher risk of having *S. aureus* infection.

Previously reported prevalence studies have shown that 20% of individuals are long-term carriers of *S. aureus* infections; about 60% are intermittent carriers and 20% rarely carry it (Chamber, 2005). Children are more likely to be persistent carriers owing to the fact that *S. aureus* is part of their normal skin flora and also present in the anterior nasal passages. The highest incidence of disease usually occurs in people with poor personal hygiene and over-crowding (Tong *et al*., 2015).

In the developing world, severe *S. aureus* infections is associated with mortality which far exceeds that in developed countries (Nickerson *et al*., 2009). Studies have reported that majority of infections in sub-Saharan countries are linked to *S. aureus* (Nantanda *et al*., 2008); consequently, *S. aureus* have been reported as the most commonly encountered bacterial species in medical microbiology laboratories within Nigeria (Ambe *et al*., 2007).

*Staphylococcus aureus* infections have increased in recent years due to resistance to commonly used drugs that are not chemically related owing to efflux pumps as the possible mechanism of resistance (Costa *et al*., 2013). Pathogenic bacteria normally possess an inclusive collection of drug efflux proteins with S. *aureus* exploiting them as a resistance method. Efflux implies "flowing out". It describes a process in which antibiotics and other biochemical constituents are expelled from a bacteria cell, resulting to resistant bacteria. Efflux mechanism is among the self-defense

mechanism employed by resistant bacteria to antibiotics thus resulting to a diminished antibiotic concentration at the site of action (Mckeegan, 2001).

Efflux pumps in bacteria may contribute to intrinsic and acquired resistance to a wide range of antimicrobial agents. Furthermore, over-expression of multidrug efflux pumps confers resistance to antimicrobial agents. Multidrug resistance (MDR) efflux is an increasingly reported occurrence with several description for certain types of micro-organisms (bacteria, fungi and protozoa) demonstrated expulsion of more than one class of antibiotics and/or other antimicrobial compounds. The multi-resistant nature of *S. aureus* may perhaps be chromosomal or plasmid related. An applicable method for eliminating resistance among bacteria; in the case of plasmid-mediated resistance is "curing" (Esebelahie *et al*., 2006). This can be achieved via the use of a chemical agent such as Rifampicin.

There is paucity of information on the prevalence of efflux pump among clinical isolates of *Staphylococcus aureus* within our environment. Therefore, this study aimed at determining the prevalence of multidrug resistant efflux pump in clinical isolates of *Staphylococcus aureus*.

## MATERIALS AND METHODS

### Isolates

One hundred and ninety-eight (198) isolates of *Staphylococcus aureus* obtained from various clinical samples (aspirates, semen, urine, catheter tips, pus and swabs) were used. The isolates were confirmed by sub-culturing on nutrient agar, incubated at 37°C overnight, and tested for catalase and coagulase. Positive catalase and coagulase isolates were confirmed as *Staphylococcus aureus*.

### Disc Susceptibility Test

Disc susceptibility test was done using British Society for Antimicrobial Chemotherapy (BSAC, 2013) method. Antibacterial discs that include: ceftazidime (30 µg), cefuroxime (30 µg), gentamicin (10 µg), ceftriaxone (30 µg) erythromycin (30 µg), cloxacillin (5 µg), ofloxacin (5 µg), amoxicillin/clavulanate (30 µg), Imipenem (5 µg) and meropenem (5 µg) were used.

### Detection of Efflux Pump

*Staphylococcus aureus* isolates that showed multiple resistance to these three antimicrobial agents (ciprofloxacin, erythromycin and gentamicin) were used. Two sets of Muller-Hinton agar plates – one containing 20mg/l of reserpine and the other set without reserpine were used. Various concentrations of the antibacterial agents ranging from 5-1280 mg/l were incorporated into both sets of media. The plates were partitioned into different sections and labelled. *Staphylococcus aureus* broth equivalent to 0.5 McFarland standard were inoculated on the plates using a standard wire loop that delivers approximately 0.01ml. Each drop contains approximately $10^6$cfu/spot. The plates were incubated at 37°C for 18-24 hrs. The minimum inhibitory concentration (MIC) assays for all antibacterial agents were determined. Presence of multi-drug resistant efflux pump was inferred when the MIC in the presence of reserpine decreased by 4- fold or more when compared with the MIC in the absence of reserpine. Two control plates were used. The first plate had neither antibacterial nor reserpine and the second plate had 20 mg/L of reserpine (Munoz-Bellido *et al*. 1999).

### Curing Experiment

Curing was performed on all *Staphylococcus aureus* isolates that exhibited multidrug resistant efflux pumps. Various concentrations of Rifampicin (40 µg) was prepared in broth and a drop of overnight broth cultures of the isolates that exhibited multidrug resistant efflux pumps added into each tube.

The minimum inhibitory concentration (MIC) of the multi-drug resistant *Staphylococcus aureus* to rifampicin was determined by the broth dilution method. The tube with highest concentration of

rifampicin that permitted growth (the next concentration after the MIC) was sub-cultured on drug-free nutrient agar plate. Disc susceptibility test was performed on exposed and unexposed multidrug resistant strains of *Staphylococcus aureus*. Loss of resistance on the rifampicin-exposed strains without concomitant loss in the unexposed strain of multidrug resistant *Staphylococcus aureus* indicates curing of plasmids (plasmid-mediated resistance) (Esebelahie *et al*., 2006).

**Ethical Approval**

An ethical approval was issued by the Hospital's Research Ethics Committee according to the Declaration of Helsinki where the study was done. Patient's consent was not required for the study.

**Statistical Analysis**

Chi ($X^2$) square test was used to determine the association of multidrug resistance and efflux pump in *Staphylococcus aureus* isolated from different specimens. The statistical software INSTAT$^R$ was used for the analysis.

**RESULTS**

The overall prevalence of multidrug resistant *Staphylococcus aureus* in this study was 32.82% with isolates from urine samples having the highest resistant strains (63.83%) than other specimens. Efflux pump was detected in 18 of the multidrug resistant *Staphylococcus aureus* isolates giving an overall prevalence of 9.09%. Isolates from urine specimens were found to be among the highest (20.28%) (Table 1).

**Table 1: Prevalence of Multidrug Resistance (MDR) and Efflux pump (EP) in *Staphylococcus aureus* isolated from different specimens.**

| Specimen | No. of isolates No. (%) | MDR isolates No. (%) | EP positive isolates No. (%) |
|---|---|---|---|
| Aspirates | 19(9.60) | 02(10.53) | 0(0) |
| Ear swabs | 18(9.09) | 03(16.67) | 0(0) |
| HVS | 50(25.25) | 08(16.00) | 1(2.00) |
| Genital ulcers | 03(1.52) | 01(33.33) | 0(0) |
| Semen | 09(4.55) | 04(44.44) | 1(11.11) |
| Urethral swab | 12(6.06) | 03(25.00) | 0(0) |
| Urine | 47(23.74) | 30(63.83) | 10(20.28) |
| Wound swabs | 27(13.64) | 0(0) | 5(18.52) |
| Pus | 11(5.56) | 13(48.45) | 0(0) |
| Catheter tips | 02(1.01) | 1(50.00) | 1(50.00) |
| **Total** | **198** | **65(32.82)** | **18(9.09)** |

Multidrug resistance: Chi-square value 43.761, $P < 0.0001$; Efflux pump: Chi-square value 24.785, $P$ - value=0.0032

KEY: No. – Number; % - Percentage; HVS – High vaginal swab

Table 2 shows resistance profile of efflux pump harboring *Staphylococcus aureus* before and after exposure to rifampicin. In all isolates there was loss of resistance to some antibacterial agents after exposure to rifampicin.

**Table 2: Resistance Profile of Efflux Pump harboring *Staphylococcus aureus* before and after Exposure to Rifampicin**

| Isolate serial Number | Resistance profile | |
|---|---|---|
| | Before exposure | After Exposure |
| 01 | Aug, Cn, Ipm, Lev, Ofl, Caz, Ery, Cip | Lev, Ofl, Caz, Cip, |
| 02 | Ery, Cip, Aug, Cn, Ofl | Ofl, Cip |
| 03 | Ofl, Ctx, Cn, Aug, Ery, Cip | Ofl, Ctx |
| 04 | Cn, Ery, Aug, Caz, Ctx, Cip | Cip |
| 05 | Aug, Ery, Cn, Ctx, Caz, Cip, Cn | Ery, Caz, Aug |
| 06 | Ipm, Aug, Ctx, Caz, Ery, Cip, Cn | Ctx, Ipm, Caz |
| 07 | Caz, Ctx, Aug, Cn, Ery, Cip | Ctx |
| 08 | Lev, Ofl, Aug, Cn, Cip, Ery | Lev, Ofl |
| 09 | Ctx, Lev, Aug, Cn, Ery | Lev, Ctx |
| 10 | Caz, Aug, Cn, Ery, Ctx | Cn, Caz |
| 11 | Cn, Ery, Caz, Cip, Aug | No Resistance |
| 12 | Cxm, Caz, Ery, Ipm, Aug, Cn | Caz, Ipm |
| 13 | Ipm, Lev, Cn, Aug, Ery | Lev, Ipm |
| 14 | Lev, Ipm, Aug, Ery, Ofl, Cn | Ofl, Aug, Lev, Ipm |
| 15 | Ipm, Aug, Ery, Ctx, Cn | Ipm, Ctx |
| 16 | Ery, Aug | No resistance |
| 17 | Cip, Ofl, Aug, Cn, Ery | Cip |
| 18 | Ofl, Cip, Aug, Cn, Ery | Ofl |

KEY: Aug- amoxicillin/clavulanate, Cn- cloxacillin, Ipm- Imipenem, Ofl- ofloxacin, Caz- ceftazidime, Ery- erythromycin, Cip- ciprofloxacin, Ctx- ceftriaxone, Cxm- cefuroxime

Of the 18 *Staphylococcus aureus* isolates that harbored efflux pump, only one utilized erythromycin alone as substrates and was cured after exposure to rifampicin. Five out of the 6 (83.33%) isolates harboring efflux pump that utilized gentamicin and erythromycin as substrates were cured of their resistance after exposure to rifampicin. Five of the 11 (45.45%) isolates with efflux pump requiring gentamicin, erythromycin and ciprofloxacin were cured after exposure to rifampicin (Table 3).

**Table 3: Distribution of Plasmid Mediated Efflux Pump in Isolates of *Staphylococcus aureus* exhibiting Efflux Pump**

| | Exposure to Rifampicin | |
|---|---|---|
| **Antibacterial agent** | **Before Exposure (%)** | **After Exposure (%)** |
| Erythromycin | 01 (100) | 01 (100 ) |
| Gentamycin | 0 (0) | 0 (0) |
| Ciprofloxacin | 0 (0) | 0 (0) |
| Erythromycin, Gentamycin | 06 (33.33) | 05(83.33) |
| Erythromycin, Ciprofloxacin | 0 (0) | 0 (0) |
| Gentamycin, Ciprofloxacin | 0 (0) | 0 (0) |
| Erythromycin, Gentamycin, Ciprofloxacin | 11 (61.11) | 05 (45.45) |

## DISCUSSION

A total of 198 clinical isolates of *Staphylococcus aureus* were used for this study. A significant number (23.74%) of the isolates were from urine specimens. This agrees with the findings of Akujobi *et al.,* 2013; Mofolorunsho *et al*. (2015) which recorded high number of *S. aureus* from urogenital and urine specimens respectively. Wound and urine formed the largest group of specimen received in Medical Microbiology Laboratories in Nigeria (Ogbolu, 2013).This may explain the observation in this study.

The susceptibility profile of the isolates to the tested antibiotics ranged from 2.53%–32.32%. Imipenem was the most active antibacterial agent (32.32%), while levofloxacin and erythromycin were the least active; with 2.53% of the *S. aureus* isolates susceptible to them. This does not agree with the findings of Okon *et al*., (2013) and Mofolorunsho *et al*., (2015) where susceptibility ranged from 14.6%-100% depending on the drug. The difference in our study and that of Okon *et al*., (2013) and Mofolorunsho *et al*., (2015) could be due to sample size and number of study area as Okon *et al*., (2013) study was carried out among six healthcare facilities in Northern Nigeria with 96 *S. aureus* isolates sampled in total, while Mofolorunsho *et al*., (2015) study was conducted among 100 *S. aureus* isolates from three healthcare facilities in Anyigba, Nigeria.

The multiple antibiotic resistance index (MARI) is a tool that divulges the extent of bacteria resistance in a given population. A multiple antibiotic resistance index ≥ 0.2 implies that the strains of such bacteria originates from an environment in which they have been exposed to a number of antibiotics (Ehinmidu, 2003). In this study, the MARI of the *S. aureus* isolates generally was ≥ 0.2, indicating that majority of the isolates has been previously exposed to antibiotics tested against in this study. The unregulated use of antibiotics in Nigeria may explain this finding. In Nigeria antibiotic use is unregulated and over the counter sales of antibiotic without prescriptions are rife (Ogbolu, 2013). This is corroborated with high MARI of *S. aureus* recovered from the nose of apparently healthy individuals in a recent study (Akinjogula *et al*., 2014).

*Staphylococcus aureus* recovered from urine were significantly ($P< 0.0001$) more multidrug resistant. Urine and wound formed the largest group of specimen received in Microbiology laboratories in Nigeria (Ogbolu, 2013), and *S. aureus* has been reported as the predominant uropathogen among asymptomatic individuals in Benin City, Nigeria (Omoregie *et al*., 2008). This suggests that in our environment, there is unregulated use of antibiotics employed in the treatment of urinary tract infections; consequently, *Staphylococcus aureus* strains exposed to these antimicrobial agents may develop resistance against them thereby becoming multi-resistant thus, explains our results in this study. As a consequence of this one may surmise that *S. aureus* strains from urine may develop multi resistant efflux pump as a means of survival.

Indeed strains of *S. aureus* isolated from urine in this study had significantly ($P=0.0032$) higher prevalence of multidrug resistant (MDR) efflux pump compared to *S. aureus* isolates recovered from other specimens. A good percentage of the isolates were multi-resistant to the drugs tested with efflux pumps been detected in 20.23% of these. These goes to show that efflux pumps are additional mechanisms by which some *S. aureus* develop resistance to drugs.

Exposure of the *S. aureus* isolates that harbored multidrug (MDR) efflux pump to Rifampicin showed loss of antibiotic resistance. Rifampicin has been used as an agent for curing resistant plasmids (Esebelahie *et al*., 2006). Resistance to some antibacterial agents detected in this study were lost after exposure to rifampicin. This indicates that their resistances were plasmid-mediated and the individual strains of *S.*

*aureus* possess multiple plasmids that coded for resistance. Nevertheless, some of the isolates with efflux pump still remained resistant after curing indicating that either they were controlled by chromosomal gene or that the efflux pumps were not inhibited by reserpine; however, reserpine has been shown not to inhibit all types of efflux pumps (Ribera *et al*., 2002).

## CONCLUSION

All isolates of *Staphylococcus aureus* examined in this study were multi-resistant with multiple antibiotic resistant index ≥ 0.2. Multidrug resistant efflux pump prevalence of 9.09% was observed and 61.11% of these were eliminated by exposure to rifampicin. In view of this study, it is recommended that combination therapy of both rifampicin and antibacterial agents with recorded plasmid mediated resistance by *S aureus* will aid for the treatment of infections caused by this highly resistant organism. In such a situation, the rifampicin cures the plasmids responsible for the resistance while the other antibacterial will eliminate the pathogen. Prudent use of antimicrobial agent is advocated to stem the tide of high bacterial resistance. Public enlightenment should be done regularly on the proper use of drugs to curb drug overuse.

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