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Beta-Lactamase Production in *Staphylococcus Aureus* from Urine of Symptomatic and Asymptomatic Subjects in Keffi, Nigeria

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Abstract: *Staphylococcus aureus is a common pathogen associated with urinary tract infection. The production of beta-lactamase by S. aureus from urine of symptomatic and asymptomatic subjects in Keffi, Nigeria was investigated. Urine samples were collected from patients with suspected cases of urinary tract infection (UTI) attending two health facilities in Keffi; and asymptomatic volunteering students, staff and others within and around the Nasarawa State University Keffi main campus. Staphylococcus aureus were isolated from the urine samples using standard cultural, microscopical and biochemical methods. Antibiotic susceptibility testing and minimum inhibitory concentration (MIC) were evaluated as described by the Clinical and Laboratory Standards Institute (CLSI). Beta-lactamase production was evaluated by iodometric methods. A total of hundred (100) isolates (50 symptomatic, 50 asymptomatic) were isolated from the urine samples. Symptomatic isolates had susceptibility which decreased as follows: streptomycin (86%) > gentamicin (84%) and erythromycin (84%) > rifampicin (80%) > norfloxacin (78%) > amoxicillin (70%) and chloramphenicol (70%) > levofloxacin (68%) > ciprofloxacin (60%) > ampicillin (58%). Asymptomatic isolates had susceptibility which decreased as follows: gentamicin (92%), streptomycin (92%) and rifampicin (92%) > erythromycin (88%) > ciprofloxacin (84%) > levofloxacin (80%) > norfloxacin (78%) > chloramphenicol (76%) > amoxicillin (72%) and ampicillin (72%). The differences in the susceptibility of symptomatic and asymptomatic isolates to the antibiotics tested were insignificant ($p > 0.05$).The MICs of amoxicillin against amoxicillin resistant S. aureus isolates for 50% and 90% of for symptomatic isolates were ≤ 8 µg/ml and ≤ 16 µg/ml respectively; for asymptomatic isolates, the MICs for 50% and 90% isolates were ≤ 34 µg/ml and ≤ 64 µg/ml respectively. Beta-lactamase was detected in 4 (23.5%) and 5 (35.7%) of amoxicillin resistant symptomatic and asymptomatic isolates respectively. Molecular characterization of beta-lactamase genes in these amoxicillin resistant S. aureus isolates from Keffi is on-going.*

Keyword: *Staphylococcus aureus, Antibiotic Susceptibility; Beta-lactamase; Symptomatic; Asymptomatic; Urine.*

1. INTRODUCTION

Urinary tract infection (UTI) is a notorious problem in both the community and hospital practices, affecting people of all ages and gender (Adeleke and Olarinde, 2013; Al-Jumaily *et al.*, 2012; Salem-Bekhit, 2014). *Staphylococcus aureus* has been reported as one of the etiological agents associated with UTIs that is commonly isolated from urine of both symptomatic and asymptomatic subjects (Demuth, 1979).

Before the introduction of penicillin in 1940, patients with *S. aureus* infection had a mortality of 90%; with the advent of penicillin era, remarkable improvement was observed in the recovery and survival of infected patients (Shrestha *et al.*, 2014). This historic triumph, however, was relatively short-lived-not too long after the introduction of penicillin in clinical use, due to the development of resistance to penicillin by *S. aureus*. Resistance mechanisms include enzymatic inactivation of the antibiotic (penicillinase and aminoglycoside-modification enzymes), alteration of the target with decreased affinity for the antibiotic (notable examples being penicillin-binding protein 2a of methicillin-resistant *S. aureus* and D-Ala-D-Lac of peptidoglycan precursors of vancomycin-resistant strains), trapping of the antibiotic (for vancomycin and possibly daptomycin) and efflux pumps (fluoroquinolones and tetracycline) (Pantosti *et al*., 2007).

Beta-lactamase production is a major mechanism by which *S. aureus* develops resistance to beta-lactam antibiotics (Akindele *et al*., 2010). The beta-lactamase enzymes produced by *S. aureus* confer

resistance against beta-lactam antibiotics like penicillin; and are encoded in the *blaZ* gene located on a transposable part of the large plasmid within the *S. aureus* bacterial cells (Hugo and Russel, 1986; Shrestha *et al.*, 2014). Because of its location, the gene is easily movable to surrounding cells through horizontal gene transfer.

In Nigeria, there are few reports of beta-lactamase mediated *S. aureus* resistance in human isolates (Olowe *et al*., 2007; Akindele *et al*., 2010; Atata *et al*., 2013; Torimiro *et al*., 2013). This study aimed at isolation of beta-lactamase producing *S. aureus* from urine of symptomatic and asymptomatic subjects in Keffi, Nigeria. Detection of the resistance mechanisms and their genetic basis is an important support to antibiotic susceptibility surveillance in *S. aureus*.

2. MATERIALS AND METHODS

2.1. Materials

Media used were: Mannitol Salt Agar (MSA: Oxoid LTD, Basingstoke, Hamphire, England), Nutrient Agar (NA: Oxoid LTD, Basingstoke, Hamphire, England), Mueller-Hinton Agar (MHA: Oxoid LTD, Basingstoke, Hamphire, England) and Mueller-Hinton Broth (MHB: Oxoid LTD, Basingstoke, Hamphire, England). The media was prepared in accordance with manufacturer's instruction.

Chemicals and Reagents used include: Hydrogen peroxide (Sigma-Aldrich Laborchemikalien GmbH), Ethanol (Sigma-Aldrich Laborchemikalien GmbH), Na_2HPO_4 (Encor Biotechnology Inc. Gainsville, Florida), Magnesium Sulphate (Nen Tech Ltd, United Kingdom), Phenol Red (HiMedia Laboratories, Marg, Mumbai, India), $NaHCO_3$, Calcium Chloride (BDH Laboratory supplies, Poole, BHIS Ltd, United Kingdom), Iodine reagent (HiMedia Laboratories, Marg, Mumbai, India), Potassium Chloride (BDH Laboratory supplies, Poole, BHIS Ltd, United Kingdom) and Potassium Iodide and soluble Starch (HiMedia Laboratories, Marg, Mumbai, India).

The antibiotic discs used were products of Optun Lab. Ltd (Nigeria); and include: Ciprofloxacin (10 µg), Norfloxacin (10 µg), Gentamicin (10 µg), Amoxicillin (20 µg), Streptomycin (30µg), Rifampicin (20 µg), Erythromycin (30 µg), Chloramphenicol (30 µg), Ampicillin (20 µg) and Levofloxacin (20 µg). Benzylpenicillin (Britannia Pharmaceutical, United Kingdom) and amoxicillin (Uttar Pradesh, India) injection powders were purchased from Pharmacy Department Federal Medical Centre, Keffi, Nigeria.

2.2. Study Area and Sample Collection

This study was carried out in Keffi metropolis. Keffi is about 53km away from the Federal Capital Territory, Abuja and 137 Km away from Nasarawa State Capital, Lafia. Keffi is located at Longitude 8'5°S (South) and Latitude 7'5°N (North) and is 630 m above sea level (Akwa *et al.*, 2007).

A total of 200 urine samples were collected- 100 from patients with UTI symptoms attending Federal Medical Center, Keffi and South Atlantic Petroleum Medical Center, Nasarawa State University, Keffi; 100 from volunteering asymptomatic students, staff and others within and around Nasarawa State University, Keffi. The samples were transported to Microbiology Laboratory, Nasarawa State University, Keffi for analysis.

2.3. Isolation of *Staphylococcus aureus*

Staphylococcus aureus was isolated from the urine of asymptomatic subjects as follows: a loopful of urine was streaked on MSA plate and incubated at 37°C for 24 h. Golden yellow colonies that grew on MSA were presumptively selected as *S. aureus.*

2.4. Identification of *Staphylococcus aureus*

The presumptive *S. aureus* was further confirmed by Gram staining of the golden yellow colonies from MSA and minimal biochemical tests for *S. aureus* identification (catalase, coagulase) as earlier described (Cheesbrough, 2006).

2.5. Antibiotic Susceptibility Testing

Antibiotic susceptibility testing of the bacterial isolates was carried out using Kirby-Bauer disk diffusion method as modified by Clinical and Laboratory Standards Institute (CLSI, 2014). Briefly four (4) colonies of the isolates were transferred into 5 ml of sterile normal saline (0.9 g NaCl, distilled water to 100 ml) in a tube such that the turbidity of the bacterial suspension was equivalent to

0.5 McFarland Standard. The sterile swab was dipped in the bacterial suspension and streaked on MHA and each antibiotic disc was aseptically placed with a sterile pair of forceps on the surface of the inoculated MHA plate. The plate was incubated at 37ºC for 24 h. The diameter of the zone of inhibition was measured using meter rule and the result was interpreted in accordance with the susceptibility break point as earlier described (CLSI, 2014).

2.6. Determination of Minimum Inhibition Concentration

The MICs of amoxicillin against amoxicillin resistant *S. aureus* were evaluated using the agar dilution method in accordance with the guidelines by the Clinical and Laboratory Standards Institute (CLIS, 2014). Briefly, two-fold concentration agar dilutions of ceftazidime or cefotaxime in MHA were prepared and inoculated with approximately 1 x 10^4 colony-forming units (CFU) from an adjusted suspension of a test organism. Results were observed and registered after incubation at 37°C for 24 h. MIC was defined as the lowest concentration that inhibited visible growth.

2.7. Detection of β-Lactamase Production

The detection of beta-lactamase production by amoxicillin resistant *S. aureus* was carried out using iodometric following a method described by Atata *et al.* (2013). Briefly, some colonies of amoxicillin resistant *S. aureus* isolates were streaked on Nutrient agar plates containing 2% (w/v) soluble starch and incubated at 37^{o}C for 24 h and the overnight agar surface was flooded with freshly prepared 10,000 unit/ml of Penicillin G (0.06 mg/ml in 0.1 M phosphate buffer, pH 7.0) and left for 15-60 min at room temperature. Thereafter, iodine solution was added. Isolates whose colonies turned blue-black with colorless halos were considered as beta-lactamase producing isolates.

2.8. Statistical Analyses

The data obtained from this study were analyzed by One way Analysis of Variance (ANOVA) using Smith Statistical Package (SSP), version 2.8 (September 26, 2005, copyright ©1995-2005 by Gary Smith, Pomona College, Claremont, California); and the significance of differences was determined at 5% probability.

3. RESULTS

3.1. Antibiotic Susceptibility

The antibiotic susceptibilities of *S. aureus* isolates from urine of symptomatic and asymptomatic subjects are as shown in Table 1. Susceptibility of symptomatic isolates decreased as follows: streptomycin (86%) > gentamicin (84%) and erythromycin (84%) > rifampicin (80%) > norfloxacin (78%) > amoxicillin (70%) and chloramphenicol (70%) > levofloxacin (68%) > ciprofloxacin (60%) > ampicillin (58%). Susceptibility of asymptomatic isolates decreased as follows: streptomycin (92%), rifampicin (92%) and gentamycin (92%) > erythromycin (88%) > ciprofloxacin (84%) > levofloxacin (80%) > norfloxacin (78%) > chloramphenicol (76%) > amoxicillin (72%) and ampicillin (72%). The differences in the susceptibilities between symptomatic and asymptomatic isolates were insignificant ($p > 0.05$).

Table1. *Antibiotic susceptibility of Staphylococcus aureus isolates from urine of symptomatic and asymptomatic subjects in Keffi, Nigeria*

Antibiotics	**Disc Content (µg)**	**No. (%) susceptibility of *S. aureus***	
		Symptomatic (n=50)	**Asymptomatic (n=50)**
Ciprofloxacin (CPX)	10	30(60)	42(84)
Norfloxacin (NB)	10	39(78)	39(78)
Gentamycin (GN)	10	42(84)	46 (92)
Amoxicillin (AMX)	20	35(70)	32(72)
Streptomycin (S)	30	43(86)	46(92)
Rifampicin (RD)	20	40(80)	46(92)
Erythromycin (E)	30	42(84)	44(88)
Chloramphenicol (CH)	30	35(70)	38(76)
Ampicillin (APX)	20	29 (58)	36(72)
Levofloxacin (LEV)	20	34(68)	40(80)

3.2. Antibiotic Resistance Phenotypes

Resistance was observed to the antibiotics tested in 48 (48.0%) of the isolates. The resistant isolates were distributed into various phenotypes as shown in Table 2. The commonest phenotypes for symptomatic isolates were APX-AMX-CH and AMX-APX-NB-CH-E at 11.5% each; and CH, CPX, CPX-LEV, APX-NB-E, AMX-APX-LEV, APX-S-NB-CH and AMX-APX-S-NB-CH-CPX-E-LEV-CN at 7.7% each. For asymptomatic isolates, the commonest phenotypes were CPX (18.2%); and CH, CPX-LEV, APX-AMX-RD-S-CH-CN and AMX-APX-RD-S-NB-CH-CPX-E-LEV at 9.1% each. The differences in the pattern of resistance between symptomatic and asymptomatic *S. aureus* isolates were insignificant ($p > 0.05$).

3.3. Multiple Antibiotic Resistance (MAR) Index

Multiple antibiotic resistance (MAR), defined here as resistance to at least two antibiotics was observed in 38 (79.2%) of the combined isolates distributed as follows: 23 (88.5%) of symptomatic and 15 (68.2%) of asymptomatic isolates. All the MAR isolates, whether from symptomatic or asymptomatic subjects, had indices ≥ 0.2 as shown in Table 3, suggesting that all of them originated from environments where antibiotics were freely abused/misused (Krumpermann, 1983). For symptomatic isolates, the commonest indices were 0.2 (11.5%), 0.3 (34.6%), 0.5 (15.4%) and 0.9 (11.5%); asymptomatic isolates had the commonest indices as 0.2 (13.6%), 0.3 (18.2%), 0.6 (9.1%) and 0.9 (9.1%).

Table2. *Antibiotic resistant phenotypes of Staphylococcus aureus isolates from urine of symptomatic and asymptomatic subjects in Keffi, Nigeria*

Antibiotic Resistant phenotypes	Frequency (%) Resistance phenotypes	
	Symptomatic (26)	Asymptomatic (22)
AMX	1(3.8)	1(4.5)
CH	2(7.7)	2(9.1)
CPX	2(7.7)	4(18.2)
AMX-APX	1(3.8)	1(4.5)
CPX-LEV	2(7.7)	2(9.1)
APX-AMX-CH	3(11.5)	1(4.5)
NB-CH-LEV	1(3.8)	1(4.5)
APX-NB-E	2(7.7)	1(4.5)
AMX-APX-LEV	2(7.7)	1(4.5)
AMX-APX-NB	1(3.8)	0(0)
APX-S-NB-CH	2(7.7)	1(4.5)
AMX-APX-S-NB-LEV	1(3.8)	0(0)
AMX-APX-NB-CH-E	3(11.5)	1(4.5)
APX-AMX-RD-S-CH-CN	1(3.8)	2(9.1)
AMX-APX-RD-S-NB-CH-E-CN	1(3.8)	1(4.5)
AMX-APX-S-NB-CH-CPX-E-LEV-CN	2(7.7)	1(4.5)
AMX-APX-RD-S-NB-CH-CPX-E-LEV	1(3.8)	2(9.1)

CH= Chloramphenicol; CPX = Ciprofloxacin; NB = Norfloxacin; CN = Gentamicin; AMX = Amoxicillin; S= Streptomycin; RD = Rifampicin; E = Erythromycin; APX = Ampicillin; LEV = Levofloxacin.

Table3. *Multiple Antibiotic Resistance Index of Staphylococcus aureus isolates from urine of symptomatic and asymptomatic subjects in Keffi, Nigeria*

No. of antibiotics MAR isolate is resistant to (a)	No. of antibiotics tested (b)	MAR indices (a/b)	No. (%) MAR isolates	
			Symptomatic (n = 26)	Asymptomatic (n = 22)
9	10	0.9	3(11.5)	2(9.1)
8	10	0.8	1(3.8)	1(4.5)
7	10	0.7	0(0.0)	0(0.0)
6	10	0.6	1(3.8)	2(9.1)
5	10	0.5	4(15.4)	1(4.5)
4	10	0.4	2(7.7)	1(4.5)
3	10	0.3	9(34.6)	4(18.2)
2	10	0.2	3(11.5)	3(13.6)

3.4. Minimum Inhibitory Concentration

The MICs of amoxicillin against amoxicillin resistant *S. aureus* isolates from urine of symptomatic and asymptomatic subjects is as given in Figure 1. The MICs (extrapolated from Figure 1) of amoxicillin for 50% (MIC_{50}) and 90% (MIC_{90}) of *S. aureus* isolates from symptomatic subjects were ≤ 8.0 µg/ml and ≤ 16.0 µg/ml respectively; for asymptomatic isolates, the MIC_{50} and MIC_{90} of the isolates were ≤ 32.0 µg/ml and ≤ 64.0 µg/ml respectively. The differences in the MICs between symptomatic and asymptomatic isolates were insignificant ($p > 0.05$).

3.5. Beta-Lactamase Production

Beta-lactamase was detected in 4(23.5%) and 5(35.7%) of amoxicillin resistant symptomatic and asymptomatic isolates respectively.

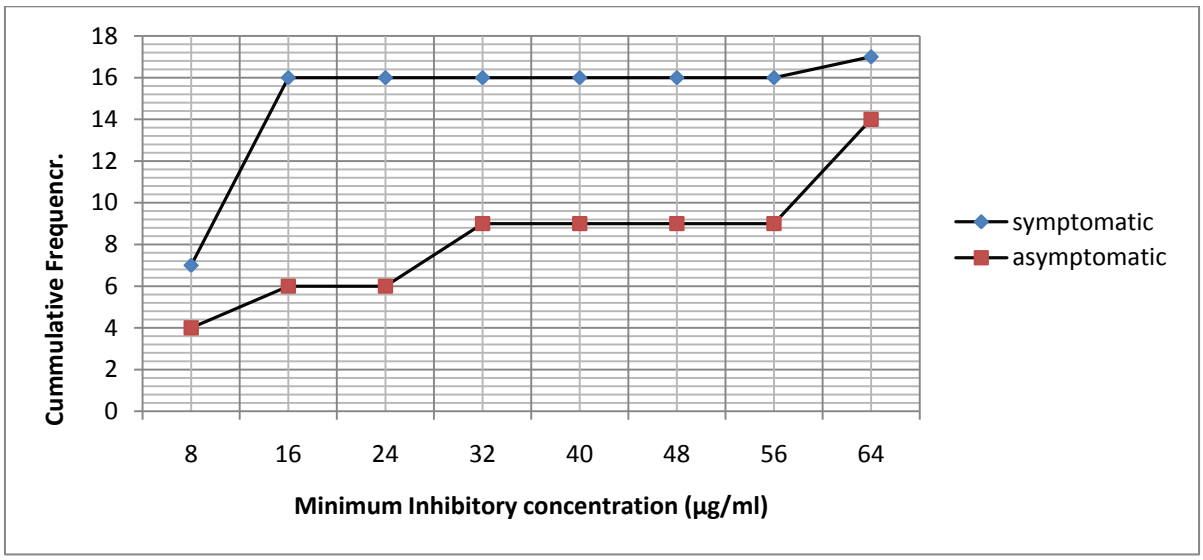

Figure1. *Minimum Inhibitory Concentration of amoxicillin against amoxicillin resistant Staphylococcus aureus from urine of symptomatic and asymptomatic subjects in Keffi, Nigeria*

4. DISCUSSION

Urinary tract infection (UTI) is a notorious problem in both the community and hospital practices, affecting people of all ages and gender (Adeleke and Olarinde, 2013). Infections caused by *S. aureus* pose serious threat in Health Care Institutions (Panlilio *et al*., 1992; NNIS, 2004). It is one of the most widely spread and virulent nosocomial pathogen and is usually resistant to multiple antibiotics making infections difficult to treat (Cooper *et al*., 2004).

This study observed a highly susceptibility of both symptomatic and asymptomatic *S. aureus* isolates from urine to the antibiotics tested. The observed high susceptibility of the isolates to chloramphenicol, ciprofloxacin and erythromycin agrees with an earlier study by Torimiro *et al*. (2013). The susceptibility to amoxicillin and gentamicin reported in this study is also in agreement with previous study conducted by other investigators (Shittu and Mandere, 1999; Olowu and Oyetunji, 2003; Akortha and Ibadin, 2008). The high susceptibility of most of the bacterial isolates to the antibiotics tested suggests misuse/abuse is minimal. For example, the discomfort of injections (e.g. gentamicin) is less likely reduce their frequently use or misuse (Ngwai *et al*., 2011); relatively higher cost of such antibiotics as ciprofloxacin in the environment under study creates affordability problem and reduces misuse. The observed high susceptibility of the *S. aureus* isolates from urine to amoxicillin and ampicillin was not expected; and is not in agreement with a previous study by Adeleke and Olarinde (2013), who reported high resistance rates by *S. aureus* against amoxicillin and ampicillin. The differences between the susceptibilities of symptomatic and asymptomatic isolates to antibiotics were insignificant.

The MIC_{50} and MIC_{90} values for the amoxicillin resistant symptomatic and asymptomatic *S. aureus* isolates was expectedly higher than the MIC breakpoint for amoxicillin described by CLSI (2014), suggesting higher level of resistance than thought from the diffusion susceptibility test results since MIC is more quantitative than normal zone of inhibition.

Beta-lactamase production can be detected in the laboratory by three different methods namely: chromatogenic, acidometric and iodometric methods (Joris *et al.*, 1994; Shrestha *et al.*, 2014). This study showed that most of the *S. aureus* isolates resistant to amoxicillin were positive for beta-lactamase production as observed in a previous study (Adeleke and Olarinde, 2013). The percentage beta-lactamase producing *S. aureus* isolates is lower than other earlier reports (Kesah *et al.*, 1997, Akindele *et al.*, 2010). Beta-lactamases are a family of enzymes produced by many bacteria that inactivates beta-lactam antibiotics by opening the beta-lactam ring (Kok *et al*., 2010). Beta-lactamases are enzymes that are responsible for many failures of antimicrobial therapy by the hydrolysis of beta-lactam ring of these antibiotics (Bush, 1989). The spread of beta-lactamase genes had been enhanced by their integration within mobile genetic elements such as plasmids and transposon which facilitate the rapid transfer of genetic materials between microbes (Wilke *et al.*, 2005). It is surprising that in this study more than half of the amoxicillin resistant strains were negative for beta lactamase production, yet were resistant to beta-lactamases. This suggests that beta-lactamase production is only a part of factors accounting for resistance in these bacteria, indicating that other mechanisms of resistance may play a major role as well. Consequently, further studies on molecular characterization of beta-lactamase production by the *S. aureus* isolates as well as other mechanisms of resistance to beta-lactam demonstrated by these isolates from urine of symptomatic and asymptomatic subjects in Keffi, Nigeria should be carried out.

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