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MOLECULAR DETECTION AND ANTIBIOGRAM CHARACTERIZATION OF *Staphylococcus aureus* STRAINS ISOLATED FROM URINE SAMPLES IN A TERTIARY HOSPITAL BASED IN BENIN CITY, NIGERIA

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ABSTRACT
The prevalence and antibiotic susceptibility profiles of *Staphylococcus aureus* strains isolated from urine samples at the University of Benin Teaching Hospital (UBTH), Nigeria, was investigated. A total of 101 bacterial isolates from urine samples were collected from the Microbiology laboratory of UBTH and identified using standard microbiological techniques. Isolates identity were further confirmed by polymerase chain reaction (PCR) and subjected to antibiotic susceptibility testing according to the description of Clinical Laboratory Standard Institute (CLSI). Thirty seven of the 101 bacterial isolates (37%) were presumptively identified as *Staphylococcus aureus* using cultural/biochemical methods, out of which 16 (43%) were confirmed as strains of *Staphylococcus aureus* by PCR technique. The PCR confirmed isolates exhibited resistance to ampicillin (100%), ceftazidime (81%), cefuroxime (75%), ceftriaxone (56%) and tetracycline (50%). Multiple antibiotic resistance (MAR) ranging from 4 to 11 antibiotics with MAR indices of 0.21 to 0.57 were observed among the bacterial isolates. This study demonstrated that multidrug resistant strains of *S. aureus* were frequently associated with urinary tract infections in Benin City, Nigeria and reaffirms the hospital setting as an important reservoir for this pathogen. It is therefore incumbent on relevant stakeholders to continually and regularly place this bacterial pathogen under close surveillance with a view to curbing its spread and preserve the public health.

Keywords: *Staphylococcus aureus*; Multidrug resistance; UTIs; Infectious disease

INTRODUCTION
Urinary Tract Infection (UTI) is an infection marked by the presence and growth of microorganisms anywhere in the urinary tract including urethra, bladder, ureter, renal pelvis, or renal parenchyma (Khoshbakt *et al*., 2013; Ezeigbo *et al*., 2015*).* It is one of the most common infections experienced by humans globally, resulting in an estimated 11.3 million hospital visits with an overall cost of 1.6 billion dollars annually in the United States (Khoshbakt *et al*., 2013)**.** Meanwhile, UTI is the most commonly associated microbial infection affecting humans in Africa (Ozumba, 2005; Aiyegoro *et al*., 2007) and is of great public health concern

both in the communities and hospital environment, and across all age group and gender (Aiyegoro *et al*., 2007).

UTI may be symptomatic or asymptomatic. In many cases the infection may be accompanied by dysuria, cystitis and pyelonephritis (Karaou and Hanna, 1981). Other signs and symptoms of the disease include fever, urinary urgency, frequent urination, chills, as well as cloudy and/or malodorous urine (Kirecci et al., 2015). UTIs are responsible for considerable morbidity and when associated with urinary obstruction or renal papillary damage, it can lead to renal failure and eventual death in severe cases (Vasudevan, 2015).

Bacteria are the major aetiology responsible for more than 95% of UTIs globally (Khoshbakt *et al*., 2013), with *Escherichia coli* reported as the most prevalent cause of the infection (Aiyegoro *et al*., 2007; Akinjogunla et al., 2010). Other bacteria incriminated in UTI include *Staphylococcus* spp., *Klebsiella* spp., *Proteus* spp., *Streptococcus faecalis, Pseudomonas aeruginosa, C. Fruendi* and *Serratia marcescens* (Abubakar, 2009). Although *E. coli* is widely reported as the most common pathogen associated with UTI (Aiyegoro *et al*., 2007; Akinjogunla *et al*., 2010; Al-Jebouri and Mdish, 2013; Vasudevan, 2015), recent studies suggests the increasing prevalence of *S. aureus* in UTIs. Raja and John (2015), reported *S. aureus* as one of the most common bacterium associated with UTIs and the third most common cause of hospital associated bacteraemia. *S. aureus* was also reported as the main aetiological agent of many infections in sub-Saharan Africa and one of the most frequently encountered bacterial species in microbiology laboratories in Nigeria (Shittu et al., 2011). Interestingly, some studies have further reported a higher proportion of *S. aureus* in UTIs in Africa. For example, 6.3–13.9% of UTIs were reportedly caused by *S. aureus* in Senegal (Dromigny *et al*., 2002), Ghana (Adjei and Opoku, 2004), and Nigeria (Otajevwo, 2013), as compared with 1.06% in Europe and Brazil (Naber et al., 2008).

Staphylococcus aureus is a Gram positive aerobe that has been implicated as the most notorious organism associated with nosocomial infections (Odjadjare and Ahmed, 2016). Although being a normal flora of the skin, *Staphylococcus aureus* when opportuned causes a number of infections (Vasudevan, 2015). It is a versatile human pathogen associated with high morbidity and mortality rates world over (Mofolorunsho et al., 2015). In the developing world, mortality associated with severe *Staphylococcus aureus* infections far exceeds that reported in developed countries (Mofolorunso *et al*., 2015). Infections caused by *S. aureus* like many other bacterial infections are usually managed and treated with antibiotics. Penicillin was the first antibiotics used for the treatment of staphylococcal infections; however penicillin resistance appeared shortly after its introduction; followed by resistance to cotrimoxazole, ampicillin, amoxicillin and cefuroxime (Raja and John, 2015). At the moment multidrug resistant (MDR) *S. aureus* is the order of the day.

The emergence of MDR *S. aureus* in the last decade has rendered the pathogen relatively more virulent,

resulting in continuous and increased incidences of *Staphylococcus aureus* infections worldwide (John and Sentry, 2004). The organism constantly evolves resistance mechanisms against existing anti-microbial agents due to its high rate of genetic plasticity; thereby necessitating an increased focus on the pathogen with a view to controlling its spread in hospitals and health care environment (Odjadjare and Ahmed, 2016). *S. aureus* has shown acquired resistance to many structurally unrelated antibiotics; and as rapidly as new antibiotics are introduced, so too the organism develops new mechanisms of resistance to such antibiotics (Brown and Ngeno, 2007). This represents a serious public health concern in terms of therapeutic options available to clinicians in managing *S. aureus* infections (Tiwari *et al*., 2008). It is therefore not surprising that *S. aureus* is increasingly incriminated in morbidity and mortality especially in developing countries like Nigeria.

Most studies on UTIs have concentrated on the antimicrobial resistance profile of Gram-negative enterobacteria especially *E. coli*, whereas the antibiotic susceptibility profile of associated Gram-positive organisms such as *S. aureus* were treated as less important despite the increasing prevalence of this organism in UTIs and its indisputable status as an epitome of multidrug resistance (Schaumberg *et al*., 2014). Policy decision on clinical management of bacterial UTIs, especially those associated with *Staphylococcus aureus* requires knowledge of its prevalence and drug susceptibility profile (Ocokoru et al., 2015). Hence the aim of this study was to investigate the prevalence and antibiotic susceptibility profiles of PCR confirmed strains of *Staphylococcus aureus* isolated from urine samples collected at the University of Benin Teaching Hospital (UBTH), Benin City, Nigeria.

MATERIALS AND METHODS

Bacterial Isolates

Bacterial isolates from urine samples were obtained from the Microbiology Laboratory of the University of Benin Teaching Hospital, Benin City, Nigeria, between January and April, 2014. A total of 101 bacterial isolates were collected under aseptic conditions and transported to the Benson Idahosa University Microbiology laboratory for analyses. Preliminary cultural/biochemical identification of *Staphylococcus* species was carried out according to the method of Bennett and Lancette (1998). *Staphylococcus aureus* ATCC 6538 was used as control organism in this study.

Molecular Confirmation of Isolates

Isolates identified as presumptive *Staphylococcus* species by cultural and biochemical methods, were confirmed by polymerase chain reaction (PCR) using the specific primers: 5'-CGCACATCAGCGTCAG-3' (reverse); 5'-GTAGGTGGCAAGCGTTATCC-3' (forward) (Anzar, 2006).

Genomic DNA Extraction

Three to five single colonies of presumptive *Staphylococcus* strains grown overnight at 37 ^{o}C on nutrient agar plates were picked, suspended in 200 µL of sterile nuclease-free water (Ambion®, Austin, Texas) in 1.5 ml Eppendorf tubes. The cells were lysed using Dri-block incubator DB.2A (Techne, Cape Town, South Africa) at

100 ^{o}C for 15 min. Cell debris were removed by centrifugation at 11, 000 × g for 2 min. using a MiniSpin micro centrifuge (Merck, Modderfontein, South Africa). The cell lysate (3 µL) was then used as template in the PCR assays immediately after extraction or following storage at -20 °C.

PCR Assay

Primer reconstitution and PCR master mix preparation were done based on manufacturers' instructions. Briefly, 25 µL solution comprising of 8.5 µL nuclease free water, 12.5 µL 2 × master mix standard buffer, 0.5 µL forward primer, 0.5 µL reverse primer and 3 µL template DNA was prepared in PCR tubes. Sterile nuclease-free water (Ambion®, Austin, Texas) was included in each PCR assay as a negative control. The thermal cycling profile was set at 94 °C initial denaturation for 5 min., followed by 30 cycles at 94 °C for 1 min., 54 °C for 1 min., 68 °C for 1 min. and final extension at 68 °C for 5 min. The amplified product was held at 4 ^{o}C after completion of the cycles prior to further analysis.

Gel electrophoresis

Ten (10) µL of DNA ladder (100 bp), nuclease free water (negative control) and PCR products of test samples (respectively) was mixed with loading dye and introduced onto wells of 1.5% agarose gel containing 0.5 mg/L ethidium bromide submerged in TAE buffer. Electrophoresis of the PCR products was carried out for 40 min. at 100 V and then visualized using a UV transilluminator.

Antibiotic Susceptibility Test

Nineteen antibiotics (Mast Group Ltd., Merseyside, UK) were used in the antibiotic susceptibility test. They include, nalidixic acid (30µg), amikacin (30µg), ceftazidime (30µg), rifampicin (5µg), cefuroxime (30µg), trimethoprim-sulfamethoxazole (1.25µg;23.75µg), trimethoprim (2.5µg), chloramphenicol (30µg), ampicillin (10µg), tetracycline (30µg), kanamycin (30µg), ceftriaxone (30µg), amoxicillin (10µg), netilmicin (30µg), ciprofloxacin (5µg), gentamicin (10µg), streptomycin (10µg), imipenem (10µg) and ofloxacin (5µg). The antibiotic susceptibility test was performed and interpreted based on the disc diffusion method described by Clinical Laboratory Standard Institute (CLSI, 2011) using Mueller-Hinton agar (Bioteck laboratories, Surrey, UK) plates. A sterile swab was placed into standardized culture of the test organism and the excess liquid was removed by gently pressing the swab against the inside of the tube. The culture was then used to swab the entire Mueller-Hinton agar plate; the plate was allowed to dry for approximately 2 min. This procedure was repeated by swabbing two more times, rotating the plate approximately 60° each time to ensure an even distribution of inoculum. Using flame-sterilized forceps, the antibiotics containing discs were pressed gently onto the agar surface to ensure that the disc adhered to the agar; the plates were incubated overnight at 37°C. The inhibition zone diameters (IZD) were measured in millimeters. Using a zone size interpretive chart (CLSI, 2011) each isolate was adjudged sensitive, intermediate or resistant to the various antibiotics as the case may be.

MAR index was calculated as described by Odjadjare *et al*. (2012) as follows:

MAR = a/b

where a = number of antibiotics to which the isolate was resistant;

b = total number of antibiotics against which individual isolate was tested.

RESULTS

A total of one hundred and one (101) bacterial isolates from urine samples were obtained from UBTH Benin City, out of which 37 (37%) were identified as presumptive *Staphylococcus aureus* using standard cultural/biochemical methods. Of the 37 presumptive *Staphylococcus aureus*, 16 (43%) were confirmed to be strains of *Staphylococcus aureus* by PCR (Figure 1). The confirmed isolates showed resistance to ampicillin (100%), ceftazidime (81%),cefuroxime (75%), ceftriaxone (56%) and tetracycline (50%); whereas they were susceptible to imipenem (100%), trimethoprim (94%), amikacin (94%), gentamicin (94%), netilmicin (94%), ofloxacin (88%), kanamycin (81%) nalidixic acid (69%), chloramphenicol (63%), and ciprofloxacin (63%) (Table 1). Multiple antibiotic resistance (MAR) ranging from 4 to 11 antibiotics were also observed among the isolates (Table 2). The MAR indices of isolates varied between 0.21 and 0.57 (Table 2).

DISCUSSION

Of the 37 presumptive *S. aureus* isolates identified using cultural/biochemical analyses, 16 (43%) were confirmed to be *S. aureus* by molecular techniques. In Nigeria, *S. aureus* strains cause significant epidemiologic and therapeutic challenges and many studies showed that identification of *S. aureus* isolates have been based largely on phenotypic methods and few data exists on the characterization of *S. aureus* isolates using molecular techniques (PCR) (Adesida *et al*., 2005; Esan *et al*., 2009). The results obtained in this study indicates that over 50% of isolates identified as *Staphylococcus aureus* by cultural and biochemical techniques may not be the organism; reaffirming the need to confirm isolates identity using molecular techniques to avoid misidentification of bacterial isolates. Misidentification of bacterial pathogens could have dire consequences on patients in terms of cost and quality of health care delivery (Esan *et al*., 2009).

S. aureus showed considerable resistance to ampicillin (100%), ceftazidime (81%), cefuroxime (75%), ceftriaxone (56%) and tetracycline (50%) (Table 1); suggesting the ineffectiveness of these antibiotics in the empiric treatment of *S. aureus* mediated UTIs. The observation is similar to the findings of Odoki *et al*. (2015) who reported resistance of UTI associated strains of *S. aureus* isolated from diabetic patients in Uganda to ampicillin, ceftazidime and ceftriaxone. In the same vein Onanuga and Awhowho (2008) reported ample resistance to ampicillin, cefuroxime and tetracycline among *S. aureus* strains isolated from UTI patients in Yenegua, Nigeria. In contrast to the observation of this study, *S. aureus* strains linked to UTI were reported to be sensitive to ceftazidime (Prakash and Saxena, 2014), cefuroxime (Ahmed *et al*., 2016), ceftriaxone (Akinjogunla *et al*., 2010; Otajevwo, 2013; Prakash and Saxena, 2014) and tetracycline (Ayepola *et al*., 2015; Onuorah and Oko, 2015).

To the best of our knowledge this is one of the very few studies that have reported the activity of imipenem against UTI associated *S. aureus* isolates in Nigeria. High sensitivity (89 to 100%) were reported in Buea, Cameroun (Longdoh *et al*., 2013), Meerut, India (Prakash and Saxena, 2014) and Moradabad, India (Jain et al., 2015) were observed against imipenem; suggesting the antibiotics was generally efficacious against UTI related *Staphylococcus aureus*. The aminoglycosides (amikacin, gentamicin, netilmicin, kanamycin and streptomycin) were also quite active against the test isolates (75 – 94%); suggesting that they are important choice drugs against *S. aureus* strains associated with UTIs in Benin City, Nigeria. The observation is similar to those reported previously (Akinjogunla *et al*., 2010; Prakash and Saxena, 2014; Ahmed *et al*., 2016) but contradicts the findings of other authors (Abubakar, 2009; Onanuga and Awhowho, 2012; Alo et al., 2015) who documented resistance against streptomycin and gentamycin among *S. aureus* strains associated with UTIs in the Nigerian cities of Yola, Yenegua and Abakaliki, respectively.

Contrary to the observation of this study, Otajevwo (2013) and Kirecci *et al*. (2015) reported high rates of resistance to ofloxacin and ciprofloxacin. This observed variance in the susceptibility pattern of the organism to the two antibiotics might be attributed to the changing nature of the pathogen in line with prevailing environmental conditions at the different locations (Alo et al., 2015). However, reports in the literature (Onanuga and Awhowho, 2008; Prakash and Saxena, 2014; Alo *et al*., 2015; Ekwealor *et al*., 2016) suggests that more often than not, *S. aureus* strains associated with UTIs were generally susceptible to ofloxacin and ciprofloxacin in agreement with the observation of this study. This observation did not come as a surprise because the two antibiotics in question are generally considered to be relatively more expensive and beyond the reach of the poor and therefore not readily subject to abuse/misuse which ultimately contributes to the development of antibiotics resistance (Udenze *et al*., 2014). The high rate of sensitivity of *S. aureus* strains in this study to nalidixic acid is one of the very few reported in recent times. Many studies over the years (Abubakar, 2009; Oluremi *et al*., 2011; Al-Jeboury and Mdish, 2013; Prakash and Saxena, 2014; Alo *et al*., 2015) have documented consistent high level resistance of *S. aureus* strains to nalidixic acid. The observation suggests that the antibiotic might be regaining its activity against *S. aureus* in agreement with the findings of Odjadjare and Ahmed (2016) who reported marginal (50%) sensitivity of *S. aureus* isolates from high vaginal swab samples to the antibiotic.

All the test isolates exhibited multiple antibiotic resistance (MAR) ranging from 4 to 11 antibiotics. The observation reaffirms the multidrug resistance prowess of *S. aureus* as corroborated by the reports of Oluremi *et al*. (2011) (3 to 8 antibiotics) and Ekwealor *et al*. (2016) (4 antibiotics). The MAR indices of the test isolates were above the 0.2 limit and suggest that the isolates originated from high risks source(s) where antibiotics are often used or abused (Odjadjare *et al*., 2012).

The observation was not surprising as the isolates were from hospital setting where antibiotics administration is a common feature and therefore creates opportunity for the development of multidrug resistance due to selective pressure (Odjadjare and Ahmed, 2016).

CONCLUSION

This study demonstrated that 50% of isolates presumptively identified as *S. aureus* using cultural/biochemical methods may not be the organism following PCR confirmation; thus reaffirming the need to always confirm bacterial isolate identity using molecular techniques such as PCR coupled with gel electrophoresis. The study also showed that MAR strains of *S. aureus* were frequently associated with urinary tract infections in Benin City, Nigeria and reaffirms that hospital environment is an important reservoir for MAR strains of *S. aureus*. There is therefore need to continually and regularly place bacterial pathogens such as *S. aureus* under close surveillance with a view to curbing their spread and preserving the public health and environment.

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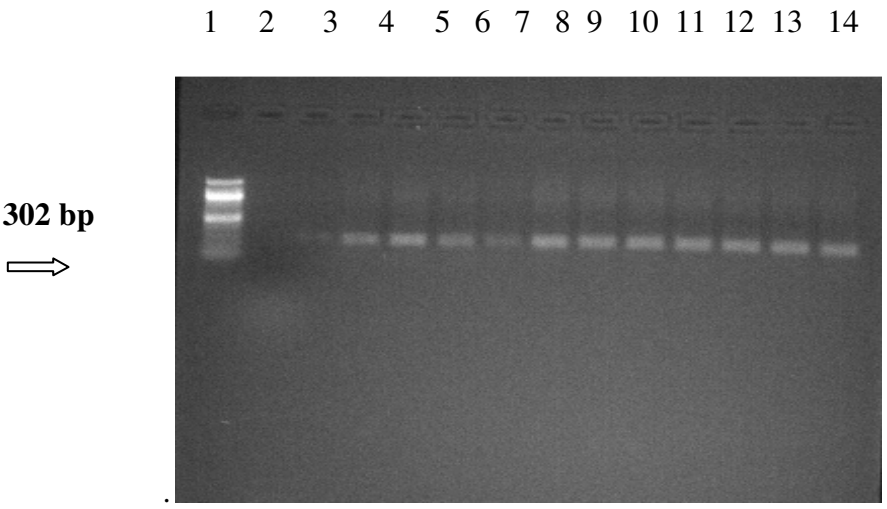

Figure 1: PCR amplicons of Staphylococcus isolates from urine samples.
Lane 1- 100bp DNA ladder; Lane 2- negative control; Lanes 3 to 14 PCR amplicons for isolates 3-U66, 4- U17, 5- U72, 6- U20, 7- U60, 8- U3, 9- U57, 10- U83, 11- U87, 12- U38, 13- U59, 14- U68

Table 1: Antibiogram of *Staphylococcus aureus* isolates from urine samples

Antibiotics	**Sensitivity (S)%**	**Intermediate (I)%**	**Resistance (R)%**
Ceftazidime	2(12.5)	1(6.25)	13(81.25)
Nalidixic acid	11(68.75)	0(0)	5(31.25)
Cefuroxime	4(25)	0(0)	12(75)
Trimethoprim	15(93.75)	1(6.25)	0(0)
Amikacin	15(93.75)	0(0)	1(6.25)
Ampillicin	0(0)	0(0)	16(100)
Trimethoprim-sulfamethoxazole	5(31.25)	11(68.75)	0(0)
Chloraphenicol	10(62.5)	5(31.25)	1(6.25)
Rifampicin	6(37.5)	7(43.75)	3(18.75)
Ciprofloxacin	10(62.5)	4(25)	2(12.5)
Kanamycin	13(81.25)	1(6.25)	2(12.5)
Amoxicillin	6(37.5)	10(62.5)	0(0)
Imipenem	16(100)	0(0)	0(0)
Ofloxacin	14(87.5)	2(12.5)	0(0)
Gentamicin	15(93.75)	1(6.25)	0(0)
Tetracycline	7(43.75)	1(6.25)	8(50)
Streptomycin	12(75)	4(25)	0(0)
Ceftriaxone	5(31.25)	2(12.5)	9(56.25)
Netilmicin	15(93.75)	0(0)	1(6.25)

Table 2: Multiple Antibiotics Resistance (MAR) profile of *Staphylococcus aureus* isolates

Isolate code	Antibiotics	MAR
1	CAZ,CXM,TM,AK,T,AP,TS,C,RP,A	0.53
2	CAZ,TM,T,AP,S	0.26
3	CAZ,CXM,TM,AP,TS,A	0.31
4	NA,CXM,TM,T,AP,TS,RP,A,NET,CRO	0.52
5	CAZ,NA,TM,T,AP	0.26
6	CAZ,CXM,TM,AP,A	0.26
7	CAZ,CXM,TM,AP,TS,A	0.31
8	CAZ,NA,CXM,TM,AP,TS,RP,CIP,A,OFX,CRO	0.57
9	CAZ,CXM,TM,AP,TS	0.26
10	TM,AP,K,S	0.21
11	CAZ,NA,CXM,TM,RP,T,AP	0.36
12	CXM,T,AP,TS,RP,S	0.31
13	CAZ,CXM,TM,AP,TS,A	0.31
14	CAZ,CXM,TM,AP,TS,RP,CIP,A,OFX,S	0.52
15	CAZ,CXM,TM,T,AP,TS,RP,CIP,A	0.47
16	CAZ,NA,TM,AP,TS,CIP	0.31
17[a]	T,CRO,TS,RP,CAZ,CXM,A,CIP,TM,AP	0.53

Legend: TET- Tetracycline, CRO- Ceftriaxone, TRM- Trimethroprim, RFP- Rifampicin, AMK- Amikacin, CAZ- Ceftazidime, CXM-Cefuroxime, NAL- Nalidixic acid, KNM- Kanamycin, CHL- Chloramphenicol, IMI- Imipenem, GEN- Gentamicin, AMX- Amoxicillin, CIP- Ciprofloxacin, TMS- Trimethoprim-sulfamethoxozole, NET- Netilimicin, AMP- Ampicillin, STP- Streptomycin, OFX- Ofloxacin. [a]*Staphylococcus aureus* ATCC 6538 (Control).