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ASYMPTOMATIC BACTERIURIA IN PREGNANCY IN OSOGBO WITH SPECIAL REFERENCE TO *STAPHYLOCOCCUS SAPROPHYTICUS*

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Abstract

Asymptomatic bacteriuria is a common clinical entity in pregnancy but the prevalence due to *S. saprophyticus*, an established uro-pathogen in sexually active women, remained largely unknown in Nigeria. The prevalence of asymptomatic significant bacteriuria due to *S. saprophyticus* was therefore determined among 431 pregnant women in a tertiary health institution, Southwestern Nigeria. Clean catch specimens of mid stream, early morning voided urine, collected on two occasions with an interval of four weeks, were subjected to microbial analysis and quantitative culture. All cultures with significant growth ($\geq 10^5$ CFU/ml) were characterized and *S. saprophyticus* identified using conventional biochemical scheme. A total of 862 duplicate urine samples were analyzed; 19.5% were positive for the same bacteria on two consecutive samples, giving a prevalence rate of 19.5% for asymptomatic bacteriuria. *S. saprophyticus* was recovered from consecutive urine of 2.6% of the women, 54.5% of whom had significant pyuria. The isolates were all susceptible to vancomycin, gentamicin and fluoroquinolones, but 18.2% were methicillin resistant strains. From the result of this study, all pregnant women should be routinely screened for *S. saprophyticus* urinary carriage.

Key words: Asymptomatic, Bacteriuria, *Staphylococcus saprophyticus*, Nigeria

Introduction

Until the 1960s, coagulase negative staphylococci (CoNS) were regarded as urinary contaminants in humans but in 1962, there was a report of isolation of CoNS possessing antigen 51 from the urine of women with acute urinary tract infection (Torres Pereira, 1962). This organism was found to belong to micrococcus subgroup 3 (Meers et al., 1975) and was later re-classified as *Staphylococcus saprophyticus* (Wallmark et al., 1978). *S. saprophyticus* is well distributed in nature and has been recovered from plant and animal food (Achi et al., 2007), and urogenital tracts of women (Rupp et al., 1992). It has been reported to be the second most frequent cause of uncomplicated UTI in young healthy women after *Escherichia coli* (Gillespie et al., 1978), but complications such as acute pyelonephritis, septicaemia, nephrolithiasis and endocarditis may occur (Raz et al., 2005).

Asymptomatic bacteriuria, defined as the presence of $\geq 10^5$ bacteria/ml of voided urine without symptoms of UTI (Connolly and Thorp, 1999), can be found in both pregnant and non-pregnant women (Olusanya et a.l, 1993), neonates (Olowu and Oyetunji, 2003), children (Kumar et al., 2002) and adult males (Nurullaev, 2004). Pregnancy is noted to enhance the progression from asymptomatic to symptomatic bacteriuria which could lead to pyelonephritis and adverse pregnancy outcomes such as prematurity and low birth weight (Connolly and Thorp, 1999), and high fetal mortality (Delzell and Leferre, 2000). This has necessitated some researchers (Aboderin et al,

2004; Taiwo et al, 2007) to suggest that all pregnant women should be routinely screened for significant bacteriuria and treated.

In Nigeria (Olusanya et al, 1993; Akerele et al, 2001; Olowu and Oyetunji, 2003; Aboderin et al, 2004; Oyagade et al, 2004; Olaitan, 2006; Taiwo et al, 2007) and other African countries (Gabre-Selassie, 1998; Turpin et al, 2007), there have been numerous studies on asymptomatic bacteriuria among pregnant women and other population groups and different prevalence rates have been reported, varying from 12% to 86% in some studies. However, most of these studies estimate significant bacteriuria based on only one urine sample and *S. saprophyticus* is rarely identified or characterized. Connolly and Thorp (1999) and Geerlings and Brouwer (2000) have defined true asymptomatic bacteriuria as two consecutive urine cultures with ≥ 10^5 CFU/ml of a bacterial species in a patient without symptoms of urinary tract infection. Our study is therefore intended to determine the prevalence of asymptomatic bacteriuria in pregnancy with special reference to *S. saprophyticus* using two urine samples collected within an interval of four weeks.

Materials and Methods

Study area

The study was carried out at Ladoke Akintola University of Technology Teaching Hospital, Osogbo, Southwestern Nigeria, over a one year period (April 2007 to March, 2008). This area is located in the tropical rain forest belt and lies approximately on latitude 40^{o}N and longitude 7.35^{o}E and 1100 meter above sea level.

Subjects

The subjects were 'apparently healthy' pregnant women on routine antenatal clinic (ANC) visit. A total of 500 subjects were recruited randomly throughout the entire period of the study. Willing participants gave informed consent and the approval of the Ethical Committee of the hospital was obtained. Subjects with ongoing clinical illness or with, obstetric complications, those with symptoms of urinary tract infection, those who submitted only one urine sample and those who or have used antibiotics for any reason within the previous two weeks were excluded. Demographic and clinical data were collected from each subject with the aid of proforma designed for that purpose.

Laboratory procedures

Sample collection

Sterile plastic containers and oral/written instructions on how to collect urine were given to the subject participants. Each subject collected 10-15 ml clean-catch mid-stream first morning urine, avoiding contamination of the container. This was transported to the Medical Microbiology laboratory of the teaching hospital, within 30 minutes of collection. Where this is not possible, participants were instructed to store the urine sample at 4^{o}C and transport to the laboratory within six hours. Urine samples were collected on two occasions from each participant with an interval of 4 weeks.

Microscopic examination of urine:

Five mls of each urine sample was centrifuged at 2,500 rpm for 5 mins. A wet film of the urine was examined microscopically under the 10x/40x objective lens for the presence of cells, casts, crystals, bacteria, trichomonads and ova of parasites (Cheesbrough, 2000).

Culture of urine

A calibrated inoculating loop (delivering 0.002mls of urine) was used to deliver a loopful of well mixed uncentrifuged urine on Cystein Lactose Electrolyte Deficient (CLED) agar (Oxoid, England). The plates were

incubated aerobically at 37°C for 24 hrs. Colonies were counted and significant bacteriuria (≥ 10^5 CFU/ml) was ascertained on any CLED agar showing ≥ 200 discrete colonies (Cheesbrough, 2000).

Isolation and biochemical identification:

Colonies suspected to be staphylococci from all culture plates with significant growth were Gram stained and subcultured on Mannitol salt agar (Oxoid, England), and incubated aerobically at 37°C for 24 hours. Colonies that were yellow (mannitol fermenting) and Gram positive cocci in clusters were tested for tube coagulase using rabbit plasma and/or for DNAse test using DNAse agar (Oxoid, England). Colonies positive on tube coagulase were confirmed as *Staphylococcus aureus*. The coagulase negative staphylococci (CoNS) were biochemically characterized using the modified scheme of Schleifer and Kloos (Schleifer and Kloos, 1975). First, all the CoNS were tested against 5µg novobiocin disk on Mueller Hinton agar (MH) and isolates with zone diameter of inhibition ≤ 16mm (novobiocin resistant) were presumptively identified as *S. saprophyticus* (Goldstein et al., 1983). This was confirmed by positive urease test and fermentation of maltose, sucrose and trehalose but not xylose, to produce acid. *S. epidermidis* was identified as coagulase negative and novobiocin sensitive (zone diameter > 16mm). Other non-staphylococcal isolates showing significant pure growth on CLED agar were also characterized to species level according to recommended techniques (Cheesbrough, 2000).

Detection of methicillin resistance:

Resistance of all the staphylococcal isolates to methicillin was determined by the oxacillin and cefoxitin disk diffusion methods. 1µg oxacillin disk was placed on MH agar supplemented with 2% sodium chloride using standard inoculum (0.5 McFarland standards ~ 1 x 10^8 CFU/ml) and incubating at 35°C for 24 hours. Oxacillin susceptibility was defined by Clinical and Laboratory Standard Institute (CLSI) criteria, (formerly called National Committee for Clinical and Laboratory Standards) (CLSI, 2005) of zone diameter of inhibition ≥ 16mm and resistance as zone diameter < 16 mm. All the isolates were also tested with 10µg cefoxitin disk on un-supplemented MH agar using the same standard inoculum (0.5 McFarland standards ~ 1 x 10^8 CFU/ml) and incubating at 35°C for 18 hours. Cefoxitin susceptibility for CoNS was based on Hederstierna-Johnsen et al (2005) criteria of zone diameter of inhibition ≥ 27mm, 22-26mm as intermediate and < 22mm as resistance.

Antibiotic susceptibility tests

Antibiotic susceptibility was performed on pure cultures of each bacteria isolate using the modified Kirby Bauer disk diffusion test (Bauer et al., 1966) on MH agar (Oxoid, England) with the following single discs; ampicillin 10µg, vancomycin 30µg, erythromycin 15µg, gentamicin 10µg, chloramphenicol 30µg, ceftriaxone 30µg and ciprofloxacin 5µg. Zone diameter of inhibition around the antibiotic disc was measured for each isolate and compared with the interpretive table of the CLSI. *S. aureus* ATCC 25923 serve as control strain for biochemical and susceptibility tests, while a resistant *S. saprophyticus* ATCC 49453 was used as control for novobiocin disc test.

Data entry and statistical analysis

Demographic and other data were manually entered into Window Vista 2007 laptop computer with GraphPad software (GraphPad Software Inc, San Diego, USA). Categorical variables were tested using Fisher exact and level of significance was set at $P < 0.05$.

Results

Of the 500 participating pregnant women, 69 were excluded, leaving 431 for analysis. The age range of the women was 16 – 45 years and mean age was 31.89±8.77 years (Table 1). A total of 862 duplicate urine samples were analyzed; 27.1% were culture positive on only one urine sample while 19.5% were positive for the

same bacteria on two consecutive samples. The prevalence of significant bacteriuria in the study, as defined by Connolly and Thorp (1999), is therefore 19.5% (84/431).

Duplicate urine samples from 11 (2.6%) women yielded significant pure growth of *S. saprophyticus*; six (54.5%) of whom had significant pyuria. Seven of the 11 *S. saprophyticus* isolates were resistant to 1µg oxacillin disk with zone diameters of inhibition varying between eight and 14 mm while only two isolates were resistant to 10µg cefoxitin disk with zone diameter of 14 mm each. The prevalence of methicillin resistance was reported as 18.2% (2/11). All the 11 isolates showed resistance to commonly available antibiotics in Nigeria such as ampicillin (63.6%), chloramphenicol (63.6%), ceftriaxone (63.6%) and erythromycin (72.7%) but were all susceptible to gentamicin, ciprofloxacin and vancomycin (Table 2).

S. aureus was isolated in 29 (6.7%) and *S. epidermidis* in 7 (1.6%). Seven of the 29 (24.1%) *S. aureus* and 4 of the 7 (57.1%) *S. epidermidis* were found to be methicillin resistant by the 10 µg cefoxitin disk. The isolates also

Table1: Distribution of pregnant women with significant bacteriuria in Ladoke Akintola University of Technology Teaching Hospital, Osogbo, Nigeria

Age group (years)	No of pregnant women	No with significant bacteriuria	Percent
16-25	15	2	13.3
26-35	172	31	18.0
36-45	244	51	20.9
Total	431	84	19.5

Age range 16-45 years; mean age 31.89±8.77years; $X^2 = 0.6246$, $P = 0.7318$

Table 2: Resistance of the staphylococcal and enterococcal urinary isolates to commonly available antibiotics in Ladoke Akintola University of Technology Teaching Hospital, Osogbo, Nigeria

Isolates	Commonly used antimicrobial agents tested (%)						
	Ampicillin	Gentamicin	Vancomycin	Erythromycin	Chloramphenico	Ceftriaxone	Ciprofloxacin
S. saprophyticus n = 11	*7 (63.6)	0	0	*8 (72.7)	*7 (63.6)	*7 (63.6)	0
S. aureus n = 29	29 (100)	18 (62.1)	0	11 (37.9)	15 (51.7)	26 (89.7)	0
S. epidermidis n = 7	7 (100)	7 (100)	0	7 (100)	0	7 (100)	0
E. faecalis n = 3	3 (100)	3 (100)	0	3 (100)	0	0	0

* Include the 2 *S. saprophyticus* isolates resistant to 10 µg cefoxitin disk

Table 3: Resistance of Gram negative bacteria urinary isolates to antibiotics in Ladoke Akintola University of Technology Teaching Hospital, Osogbo, Nigeria

Isolate	Ampicillin	Erythromycin	Tetracycline	Chloramphenicol	Gentamicin	Cotrimoxazole	Ceftriaxone	Ciprofloxacin
E. coli (n=14)	14(100)	14(100)	14(100)	14(100)	11(78.6)	14(100)	14(100)	0
Kl. aerogenes (n=20)	20(100)	20(100)	20(100)	20(100)	20(100)	20(100)	20(100)	7(35)
Ps. aerugionosa (n=4)	4(100)	4(100)	4(100)	4(100)	4(100)	4(100)	4(100)	2(50)

demonstrated high resistance rates to locally available antimicrobial agents as shown in Table 2, but all isolates were sensitive to 30 µg vancomycin disk.

Other isolates recovered from duplicate samples are *Enterococcus faecalis* 3 (0.7%), *Escherichia coli* 14 (2.8%), *Klebsiella aerogenes* 20 (4.2%) and *Pseudomonas aeruginosa* 4 (0.9%). These isolates also demonstrated high resistance to most of the antibiotics tested as shown in Table 3.

Discussion

This study recorded a 19.5% prevalence rate for asymptomatic significant bacteriuria among pregnant women in Osogbo, Southwestern Nigeria. Only asymptomatic subjects whose two consecutive urine samples cultured positive for the same pathogen were considered to have true significant bacteriuria. We observed that 28% of the pregnant women who were cultured positive with the first urine sample were not positive with the second urine sample. This is similar to what some researchers (Aboderin et al, 2004) have observed where only about 70% of pregnant women positive on first occasion remain positive the second time.

The prevalence rate of 19.5% in this study is comparatively less than the rates reported previously in some Nigerian tertiary healthcare institutions (Olusanya et al, 1993; Akerele et al, 2001; Aboderin et al, 2004; Taiwo et al, 2007), probably because we used two consecutive positive samples while those previous studies used only one. If we had considered only one sample, the rate in our study would have been about 27.1%, and this will agree with these previous rates, but will be an over-estimate of the true situation. However, our prevalence rate is still higher than those of some other health institutions in Nigeria (Onyemelukwe et al, 2003; Oyagade et al, 2004) and much higher than those from other African countries such as Ethiopia (Gabre-Selassie, 1998) with 7% and Ghana (Turpin et al, 2007) with 7.3%, and from the Western world with 2.6 - 5.1% (Campbell-Brown et al, 1987; Nicolle, 1994). This observation may be attributable to differences in socioeconomic status, level of health care development, selection of subjects, methodology and interpretation of significant bacteriuria (Onyemelukwe et al, 2003; Taiwo et al, 2007).

In this study, *S. saprophyticus* was recovered repeatedly in pure culture and high count ($\geq 10^5$ CFU/ml) from urine of 11 pregnant women all in the age group 16-40 years. None of them had symptoms or recent history of a UTI, but six of them had significant pyuria on urine microscopy (≥10 WBC/HPF). Rupp et al (1992) reported 6.9% (19/276) prevalence of *S. saprophyticus* colonization of female urogenital tracts in an outpatient gynaecology practice, which included colonization of the rectum, urethra, urine and cervix in descending order. In their study, colonized women were more likely to have experienced a UTI in the previous 12 months or recent menstrual periods but in our study, all the colonized patients denied history of UTI in the preceding year or recent menstrual periods, as most of them were in the second or third trimester of pregnancy. Although, follow-up of the patients for an average of 6.75 months failed to document any colonized woman progressing to symptomatic UTI in the Rupp et al (1992) study, *S. saprophyticus* isolates in our study cannot be dismissed as contaminants, because the organism has been implicated as the second most common cause of uncomplicated acute UTI in young sexually active healthy women in the Western world where it has mostly been characterized (Wallmark et al 1978; Gillespie et al, 1978; Raz et al., 2005). Also, the recent whole genome sequencing of *S. saprophyticus* (Kuroda et al, 2005) has thrown more light in understanding the pathogenesis of acute uncomplicated UTI caused by this organism.

The observation about *S. saprophyticus* among young women in the Western world is probably the same in developing countries, but because CoNS are often regarded as contaminants and therefore not usually characterized to species level, it has been difficult to know the true prevalence of this organism in Nigeria. The development of UTI is likely to be preceded by adherence to uro-epithelium and colonization of the bladder by *S. saprophyticus*. The finding of significant pyuria in more than 50% of the pregnant women with significant *S. saprophyticus* bacteriuria in this study, which persisted after one month of follow up, implies that this organism is an important uro-pathogen among pregnant women in Nigeria. From the foregoing, it has become necessary for microbiology laboratory to characterize CoNS in order to identify *S. saprophyticus* that may be an important pathogen causing symptomatic bacteriuria or ascending UTI in young pregnant and non-pregnant women.

S. aureus was recovered in high number of women in the study. This organism is usually the most frequently isolated Gram positive bacterium in studies of asymptomatic or symptomatic bacteriuria in Nigeria (Olusanya et al, 1993; Akerele et al, 2001; Olowu and Oyetunji, 2003; Aboderin et al, 2004; Oyagade et al, 2004;

Olaitan, 2006; Taiwo et al, 2007) and other African countries (Gabre-Selassie, 1998; Turpin et al, 2007) although its role in ascending UTI is unclear; rather, *S. aureus* UTI mostly result from the spread of organism to the kidneys from a blood stream infection. Other non-*saprophyticus* CoNS are also recovered in urine in significant proportion but uro-pathogenic role for these CoNS has not been demonstrated and they are generally regarded as urethral contaminants (Marrie et al, 1982) although a few of them have been implicated in isolated cases of cystitis (Gunn and Davies, 1988).

The characterization of *S. saprophyticus* based on susceptibility to novobiocin 5µg used in this study is an important diagnostic tool in differentiating species of CoNS (Goldstein *et al,* 1983) and has been shown to have excellent correlation with molecular methods such as the 16S - 23S rRNA intergenic spacer length polymorphism analysis by PCR (Shittu et al., 2006). The susceptibility of the *S. saprophyticus* isolates shows high resistance to commonly available antibiotics such as ampicillin, chloramphenicol, erythromycin and ceftriaxone, which are obtainable over-the-counter without medical prescription in Nigeria. However, the 1µg oxacillin disk we used to screen for methicillin resistance among the 11 *S. saprophyticus* isolates was seriously flawed as five of the seven (71.4%) isolates found to be resistant were susceptible when 10µg cefoxitin disks was employed.

Perazzi et al. (2006) have reported difficulties in detecting true oxacillin resistance mediated by PBP 2a in CoNS with 1µg oxacillin disk while Higashide et al. (2006) have reported that oxacillin has specificity of only 13% in detecting methicillin resistance in *mecA* positive *S. saprophyticus*. Five of the seven isolates resistant to 1µg oxacillin produced β-lactamase which we detected with the chromogenic cephalosporin method (data not shown). This may have interfered with oxacillin susceptibility by slowly hydrolyzing the drug thereby creating false resistance. Over-production of β-lactamase has been suggested as a major factor in false-positive detection of oxacillin resistance in CoNS (Ghoshal et al., 2004). We suggest the use of 10µg cefoxitin as a replacement to 1µg oxacillin disk in screening for methicillin resistance in CoNS especially in a resource poor country where there are no facility to perform PCR for the detection of *mecA* gene responsible for methicillin resistance in the staphylococci.

The mechanism of resistance to methicillin in the two *S. saprophyticus* isolates that were resistant to 10µg cefoxitin is unknown. We had no facility in our centre to screen them for carriage of *mecA* gene or production of PBP 2' which mediates methicillin resistance in *S. aureus* and other CoNS, and the two isolates were also negative for β-lactamase (data not shown). It has been suggested that the mechanism of oxacillin resistance in *S. saprophyticus* may be other than the production of PBP2a. A study by Shittu et al. (2006) in Ile-Ife, Nigeria also showed two *S. saprophyticus* isolated from the nose of medical personnel to exhibit resistance to oxacillin but lack *mecA* gene. In addition, whole genome sequence of *S. saprophyticus* has shown that its staphylococcus cassette chromosome (*SCC*) does not contain the *mec* antibiotic resistance determinant (Kuroda et al., 2005). However two *mecA* positive, PBP 2a negative *S. saprophyticus* were reported by Perazzi et al. (2006) to be resistant to oxacillin but susceptible to cefoxitin.

The high multi-resistance rates among the *S. saprophyticus* isolates in this study may be a direct effect of misuse and abuse of these agents by the general populace. However, the isolates were still largely susceptible to gentamicin and fluoroquinolones, which are rather too expensive or not readily available for most people to abuse in this environment. All the isolates were also sensitive to vancomycin 30µg by the disc diffusion test used in the study, although this method frequently misclassifies intermediately susceptible strain as fully susceptible (Tenover et al., 1998). Historically, the development of vancomycin resistance in staphylococci was pre-dated by the prolonged use of vancomycin in treatment of severe staphylococcal infections (Kist et al., 1998). There are no documented records of such use in Nigeria hence vancomycin resistance is not expected. The resistance of *S. aureus* and other CoNS to various antibiotics in this study parallel that for *S. saprophyticus.*

Conclusion

Asymptomatic significant bacteriuria due to *S. saprophyticus* is an important entity among pregnant women in LAUTECH Teaching Hospital. Screening of all pregnant women for this entity should be incorporated into routine antenatal practice and the microbiology laboratory should routinely characterize all significant CoNS isolates from urine to species level.

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