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ISOLATION OF MULTIPLE ANTIBIOTIC RESISTANT BACTERIAL SPECIES FROM MIDSTREAM URINE SAMPLES OBTAINED FROM CONSENTING HEALTHY FEMALE UNIVERSITY STUDENTS IN BENIN CITY, EDO STATE

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ABSTRACT
Culturable bacterial flora associated with clean catch midstream urine samples was determined using pour plate procedure. The antibiogram profile of the characterized bacterial cultures was evaluated using disc diffusion technique. The antibiotic discs utilized were; Ciprofloxacin (CIP), Cloxacillin (OB), Ofloxacin (OFX), Erythromycin (ERY), Caftazidime (CAZ), Gentamicin (CN), Cefuroxime (CXM), Ceftriaxone (CTR) and Amoxcillin Clavulanate (AM). Urine samples were obtained from thirty (30) randomly selected female students residing in two (2) female hostels located within the main campus of a private university in Benin City, Edo State. The heterotrophic bacterial count on Nutrient agar and Blood agar plates for the urine samples varied from 5 cfu to 263 cfu and 2 cfu to 220 cfu respectively. The observed difference between the urine derived bacterial counts was statistically significant ($p<0.05$). *The* differential bacterial counts on CLED plates varied from 1 cfu to 240 cfu. Five (5) bacterial isolates were identified from the urine samples and these isolates included; *Staphylococcus aureus, Escherichia coli*, *Pseudomonas aeruginosa, Klebsiella* sp. and *Proteus* sp. Aside from the *S. aureus* (U15), all the respective urine derived bacterial cultures exhibited susceptibility towards ciprofloxacin. All the bacterial strains were resistant to cloxacillin. *S. aureus* (US18) and *Klebsiella* sp. (U29) had maximal MAR value while S. *aureus* (U20) had the lowest MAR index value. The occurrence of female cases of asymptomatic bacteriuria within the relatively small population sub set should be reported to the relevant health care authorities within the institution for necessary action.

KEYWORDS: *Disc diffusion, Urine, Female, Asymptomatic Bacteriuria, Antibiotic disc*

INTRODUCTION

Urine has been described as a pale-yellow fluid produced by the kidneys and it is known to contain urea, uric acid, minerals, chloride, nitrogen, sulphur, ammonia, copper, iron, phosphate, sodium, potassium, manganese, carbolic aid, calcium, salts; vitamins A, B, C, and E; enzymes, hippuric acid, creatinine, as well as lactose (Dada and Aruwa, 2014). Urine pH has been documented to vary between 4 to 8. The bladder and urinary tract are usually sterile. The urethra however, has been known to occasionally harbor a few commensals, and also the perineum which can contaminate urine when it is being passed out. Some of these commensals include; Diphtheroids, enterobacteria, *Acinetobacter* spp. and some skin commensals such as Gram-positive staphylococci, micrococci, and Gram-positive enterococci (Dada and Aruwa, 2014). Dada and Aruwa (2016) described bacteriuria as the presence of significant bacterial count in urine. Frank-Peterside and Wokoma (2009) opined that bacteriuria usually precede symptomatic urinary tract infection, which is characterized by dysuria, increased frequency in urination, pain, and fever. Urinary tract infection (UTI) is any infection of a part of the urinary tract. UTIs are among the most common bacterial infections in human, community and hospital settings (Nicolle *et al*., 2005). It is known to occur in all age groups, genders, and usually requires urgent treatment (Orrett and Davis, 2006). Bacteriuria can be symptomatic or asymptomatic, but UTI involves clinical signs and symptoms (Frank-Peterside and Wokoma, 2009). Asymptomatic bacteriuria (AB) or urinary tract infection has been described as the isolation of a specified quantitative count of bacteria in an appropriately collected urine specimen from an individual without symptoms or signs of urinary tract infection (Dada and Aruwa, 2016) AB is common with varying prevalence by age, sex, sexual activity and the presence of genito-urinary abnormalities (Dada and Aruwa, 2016). Women with AB are more likely to experience symptomatic UTI than those without AB. *Escherichia coli* is the most frequent microorganism isolated from subjects with asymptomatic bacteriuria (Nicolle, 2003). *E. coli* has remained the single most common microorganism isolated from women, but other microorganisms, such as *Proteus mirabilis*, are more common in men (Nicolle *et al*., 2005). Motamedifar *et al*. (2016) observed that bacteriuria can be regarded as been significant when the urine contains about 10^5 organisms or more per millilitre in pure culture.

The aim of this study was to determine the identity of bacterial flora associated with fresh urine samples obtained from consenting female students. The antibiogram and multiple antibiotic resistance (MAR) index of the bacterial isolates were also evaluated.

MATERIALS AND METHODS

Study Area

The main campus of Benson Idahosa University; GPS coordinates; 06°11′20.5″N; 05°39′21.2″E located at Okha village, at the outskirts of Benin City has a relatively large student population and a sizable proportion of these students are domiciled within several hostels located within the

campus. The university is situated in the humid tropical environment. There are about four (4) female hostels located within the campus premises; old hostel A and D and Bishop's court A and C respectively.

Study Population

The study population comprised of 30 female undergraduate students of a private university located in Benin City, Edo State, and all the students were residing in both of the female hostels sited within the school main campus. Their ages ranged from 16 years to 26 years. The consenting students showed no symptoms of urinary tract infections and were not on any form of antibiotic therapy as at the time of sample collection.

Collection of Urine Samples

Ten (10) ml of clean catch midstream urine was obtained from thirty (30) undergraduate females with the aid of sterile universal bottles. Prior to urine collection, the consenting students spread the labia, and cleanse the vulva and labia thoroughly using sterile cotton gauze pads and warm soapy water respectively (Vandepitte *et al*., 2003). The students also wiped their vagina from front to rear (Vandepitte *et al*., 2003). Sampling was conducted between 7am-8am daily for the duration of two (2) months; February and March, 2019. Upon collection of the urine samples, the universal bottles were labeled and taken to the laboratory where bacteriological analysis was conducted.

Ethical Approval and Consent to Participate

As at the time of sampling, there was no functional faculty based ethical committee operating in the private university where the respective female students both attend and resided. However, informed oral consent was duly obtained from the participating student after the purpose of collecting the urine samples was explained to the consenting students.

Determination of the Culturable Heterotrophic Bacterial and Coliform Counts of the Urine Samples

The heterotrophic bacterial counts associated with the urine samples were evaluated using pour plate procedure as described by Harley and Prescott (2002). Prepared Nutrient agar (NA), Blood agar and Cystine-Lactose-Electrolyte Deficient (CLED) agar plates were utilized in the determination of the heterotrophic bacterial flora present in the urine samples. These agar plates were incubated at 35°C for 24 h and the colonial morphologies of the respective bacterial isolates were noted and recorded. Also, the sub cultured bacterial isolates were also subjected to an assortment of physiological and morphological tests which included Gram staining, catalase and oxidase production tests and the results of these procedures were collated and compared with documented identification schemes of different bacterial groups as provided by Cullimore (2000) and Cappuccino and Welsh (2020).

Determination of the Antibiotic Sensitivity Pattern (Antibiogram) of the Bacterial Isolates

The antibiotic sensitivity pattern (antibiogram) of the bacterial isolates was determined using the disc diffusion method as described by Harley and Prescott, (2002) and Vandepitte *et al*., (2003) respectively. Commercially available antibiotic discs produced by both Rapid Labs™ and Oxoid™ which contained varying concentrations of the

respective antibiotics was utilized and these discs are; Ciprofloxacin (CIP) (5 µg), Cloxacillin (OB) (5 µg), Ofloxacin (OFX) (5 µg), Erythromycin (ERY) (5 µg), Caftazidime (CAZ) (30 µg), Gentamicin (CN) (10 µg), Cefuroxime (CXM) (30 µg), Ceftriaxone (CTR) (30 µg) and Amoxcillin Clavulanate (AM) (30 µg) respectively. These plates were incubated overnight (for about 10 h). The resultant visible zones of inhibition were measured using a ruler. The measured zones were interpreted as resistant (R), intermediate (I) and susceptible (S) according to an interpretative chart of zone dimensions for rapidly growing bacteria exposed to antibiotic disc developed by National Committee for Clinical Laboratory Standards (NCCLS) as detailed by Vandepitte *et al*. (2003).

Determination of the Multiple Antibiotic Resistance (MAR) Index

The multiple antibiotic resistance index of the exposed bacterial isolates was evaluated using a formula described by Akinjogunla and Enabulele, (2010). The formula is given below

MAR: A/B

where;

A: represent the number of antibiotics to which the isolate was resistant

B: total number of antibiotics to which the isolate was exposed.

Data Analysis

The urine derived counts were subjected to non-parametric F-test analogue; Kruskal Wallis with the aid of SPSS version 22 (α=0.05) (Ogbeibu 2005).

RESULTS AND DISCUSSION

The culturable bio-load associated with the urine samples is presented in Table 1. The heterotrophic bacterial count on Nutrient agar and Blood agar plates for the urine samples varied from 5 cfu for U1 to 263 cfu for U16 and 2 cfu for U1 to 220 cfu for U20 respectively (Table 1). The differential bacterial counts on CLED plates for the respective urine samples varied from 1 cfu for U4 to 240 cfu for U12 (Table 1). The observed difference between the urine derived bacterial counts was statistically significant ($P<0.05$) (Table 1). Five (5) bacterial isolates were identified from the urine samples and these isolates included; *Staphylococcus aureus, Escherichia coli*, *Pseudomonas aeruginosa* Klebsiella sp. and *Proteus* sp. (Table 2 to 5).

All the examined urine samples had varying heterotrophic bacterial counts. This trend would suggest that all the sampled female student population had asymptomatic bacteriuria (AB). This trend was at variance with an earlier study by Frank-Peterside and Wokoma (2009) which indicated bacterial presence in 89% of the collected urine samples while there was no bacterial growth in 11% of the urine samples. The authors suggested several reasons which included; presence of intestinal bacteria or contaminants from the vagina, feces or perinial skin for the high incidence of AB among non-pregnant females. Nicolle (2003) observed that in apparently healthy women, the prevalence of bacteriuria can increase with age, from about 1 % in females, 5 to 14 years of age to more than 20 % in women at least 80 years of age living in a given community. *E. coli* was most frequently isolated bacterium from the urine samples (Tables 2 to 5b). This observation contrasted with the report by Frank-Peterside and Wokoma

(2009) which detailed a higher prevalence of *S. aureus* amongst urine associated bacterial cultures.

All the bacterial strains recovered from the urine samples were resistant to cloxacillin (Table 2, 5a and 5b). Aside from the *S. aureus* strain isolated from U15, all the respective urine derived bacterial cultures exhibited susceptibility towards ciprofloxacin (Table 2 and 5b).

With the exception of *S. aureus* isolated from U15, U20 and U21, all the *S. aureus* strains were resistant to erythromycin (Table 2 and 5b). Ciprofloxacin was the most effective antibiotic against the HVS derived isolates as about 96.875 % of the exposed isolates exhibited sensitivity toward this drug (Table 2). With the exception of *P. aeruginosa*, all the bacterial isolates were susceptible to ofloxacin (Table 2 and 3). Aside from *S. aureus* U8 strain, all the urine derived bacterial cultures were resistant to erythromycin (Table 2).

Amongst the bacterial isolates, *S. aureus* (US 18) and *Klebsiella* sp. (U29) had maximal MAR value while S. *aureus* (U20) had the lowest MAR index value (Table 5a and 5b). There were variations in the antibiogram profiles of the characterized urine borne bacterial strains. It is difficult to suggest possible reasons for these variations especially as the exposed bacterial strains are endogenous to the FGT (female genital tract) of the individuals and information pertaining to the consumption pattern of over-the-counter antibiotics by the consenting females was unavailable as at time of urine collection. It has been documented that bacterial susceptibility or resistance to a particular antibiotic is intrinsic. However, bacterial resistance to antibiotics can be acquired genetically and expressed phenotypically in several ways which include; enzymatic degradation of antibiotics, antibiotics target modification, change of bacterial cell wall permeability and alternative pathways to escape the effect of antibiotics (Verraes *et al*., 2013). The sensitivity of the urine sourced bacterial strains to gentamicin is in agreement with a report by Nwogwugwu *et al*. (2015) which documented a similar trend. Worryingly, *S. aureus*, *Klebsiella* sp., *E. coli, Proteus* sp. and *Klebsiella* sp. strains exhibited high MAR indices which could suggest the potentials of these commensals to cause opportunistic infections within the FGT of the affected individuals.

Table 1: Heterotrophic and differential bacterial counts of the urine samples after 24 hours

Sample code/ Statistical inference	Bacterial counts on NA (cfu)	Bacterial counts on BA (cfu)	Differential bacterial counts on CLED (cfu)
U1	5	2	0
U2	46	10	28
U3	0	4	0
U4	27	9	1
U5	90	60	32
U6	50	26	9
U7	8	0	0
U8	7	15	20
U9	11	0	12
U10	0	0	125
U11	0	0	15
U12	164	215	240
U13	142	38	0
U14	67	220	156
U15	135	37	42
U16	263	15	120
U17	84	12	53
U18	133	50	17
U19	21	46	32
U20	120	205	123
U21	102	82	12
U22	7	14	18
U23	48	0	20
U24	38	25	15
U25	31	10	30
U26	18	8	7
U27	41	15	8
U28	12	19	6
U29	33	14	11
U30	8	6	5
P value	.001	.001	.001
Significance	*P<0.05*	*P<0.05*	*P<0.05*

Key: NA, Nutrient Agar; CLED, Cystine Lacose Electrolyte Deficient agar; BA, Blood Agar

Table 2: Antibacterial susceptibility pattern (percentage) of the urine derived bacterial isolates (N=32)

Bacterial isolates	No. of isolates	CIP	OB	CN	CTR	ERY	OFX	AM	CAZ	CRX
E. coli	11	11(100)	0 (0)	11 (100)	0(0)	0(0)	11(100)	11(100)	0(0)	0 (0)
Klebsiella sp.	5	5 (100)	0 (0)	5 (100)	3 (60)	0(0)	5 (100)	0(0)	0(0)	2 (40)
S. aureus	6	5 (83.3)	0 (0)	6 (100)	3 (50)	3(50)	6 (100)	3(50)	3(50)	6(100)
Proteus sp.	6	6 (100)	0(0)	6(100)	0(0)	0(0)	6(100)	6(100)	0(0)	0 (0)
P. aeruginosa	4	4 (100)	0 (0)	4 (100)	4 (100)	0 (0)	0 (0)	4 (100)	4(100)	0 (0)
Total	32	31 (96.875)	0(0)	32 (100)	10(31.25)	3(9.375)	28(87.5)	24 (75)	7(21.875)	8 (25)

KEY: N; Number, (%), Ciprofloxacin (CIP), Cloxacillin (OB), Ofloxacin (OFX), Cefuroxime (CRX), Erythromycin (ERY), Caftazidime (CAZ), Gentamicin (CN), Ceftriaxone (CTR) and Amoxcillin Clavulanate (AM)

Table 3: Antibacterial resistance pattern (percentage) of the urine derived bacterial isolates (N=32)

Bacterial isolates	No. of isolates	CIP	OB	CN	CTR	ERY	OFX	AM	CAZ	CRX
E. coli	11	0 (0)	11(100)	0 (0)	5(45.45)	11(100)	0 (0)	0(0)	11(100)	11 (100)
Klebsiella sp.	5	0 (0)	5 (100)	0 (0)	0 (0)	5(100)	0 (0)	0(0)	5(100)	0 (0)
S. aureus	6	1 (16.6)	6 (100)	0 (0)	3(50)	3 (50)	0 (0)	3(50)	3(50)	0 (0)
Proteus sp.	6	0(0)	6 (100)	0(0)	6(100)	6(100)	0(0)	0(0)	3(50)	6 (100)
P. aeruginosa	4	0 (0)	4 (100)	0 (0)	0 (0)	4(100)	4(100)	0 (0)	0 (0)	4(100)
Total	32	1 (16.6)	32 (100)	0 (0)	14(43.75)	29(90.625)	4 (12.5)	3(9.375)	22(68.75)	21 (65.625)

KEY: N; Number, (%), Ciprofloxacin (CIP), Cloxacillin (OB), Ofloxacin (OFX), Cefuroxime (CRX), Erythromycin (ERY), Caftazidime (CAZ), Gentamicin (CN), Ceftriaxone (CTR) and Amoxcillin Clavulanate (AM)

Table 4: Intermediate antibacterial susceptibility or resistance pattern (percentage) of the urine derived bacterial isolates (N=32)

Bacterial isolates	No. of isolates	CIP	OB	CN	CTR	ERY	OFX	AM	CAZ	CRX
E. coli	11	0 (0)	0 (0)	0 (0)	6(54.54)	0 (0)	0 (0)	0(0)	0 (0)	0 (0)
Klebsiella sp.	5	0 (0)	0 (0)	0 (0)	2 (40)	0 (0)	0 (0)	5(100)	0 (0)	3(60)
S. aureus	6	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0(0)	0 (0)	0 (0)
Proteus sp.	6	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	3(50)	0(0)
P. aeruginosa	4	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Total	32	0 (0)	0 (0)	0 (0)	8(25)	0 (0)	0 (0)	5(15.625)	3(9.375)	3(9.375)

KEY: N; Number, (%), Ciprofloxacin (CIP), Cloxacillin (OB), Ofloxacin (OFX), Cefuroxime (CRX), Erythromycin (ERY), Caftazidime (CAZ), Gentamicin (CN), Ceftriaxone (CTR) and Amoxcillin Clavulanate (AM)

Table 5a: MAR index of the urine associated bacterial cultures

Strain (source)	CIP (5 µg)	OB (5 µg)	CN (10 µg)	CTR (30 µg)	ERY (5 µg)	OFX (5 µg)	AM (30 µg)	CAZ (30 µg)	CRX (30 µg)	MAR index
E. coli (U 02)	S	R	S	I	R	S	S	R	R	0.4
E. coli (U04)	S	R	S	R	R	S	S	R	R	0.5
E. coli (U 11)	S	R	S	R	R	S	S	R	R	0.5
E. coli (U12)	S	R	S	R	R	S	S	R	R	0.5
Klebsiella sp. (U 06)	S	R	S	S	R	S	I	R	S	0.3
Klebsiella sp. (U 07)	S	R	S	I	R	S	I	R	S	0.3
Proteus sp. (U 1)	S	R	S	R	R	S	S	R	R	0.5
Proteus sp. (U 20)	S	R	S	R	R	S	S	R	R	0.5
P. aeruginosa (U09)	S	R	S	S	R	R	S	S	R	0.4
P. aeruginosa (U 14)	S	R	S	S	R	R	S	S	R	0.4
S. aureus (U 05)	S	R	S	S	R	S	S	S	S	0.2
S. aureus (U 08)	S	R	S	R	S	S	R	R	S	0.4

KEY: S: Sensitive, R: Resistant, I: Intermediate, MAR; Multiple antibiotic resistance, Ciprofloxacin (CIP), Cloxacillin (OB), Ofloxacin (OFX), Cefuroxime (CRX), Erythromycin (ERY), Caftazidime (CAZ), Gentamicin (CN), Ceftriaxone (CTR) and Amoxcillin Clavulanate (AM)

Table 5b: MAR index of the urine associated bacterial cultures contd.

Strain (source)	CIP (5 µg)	OB (5 µg)	CN (10 µg)	CTR (30 µg)	ERY (5 µg)	OFX (5 µg)	AM (30 µg)	CAZ (30 µg)	CRX (30 µg)	MAR index
E. coli (U 24)	S	R	S	I	R	S	S	R	S	0.3
E. coli (U 20)	S	R	R	R	R	S	S	R	S	0.5
E. coli (U21)	S	R	S	I	R	S	S	R	R	0.4
E. coli (U22)	S	R	S	S	R	S	S	R	I	0.3
E. coli (U23)	S	R	R	R	R	S	S	R	I	0.5
E. coli (U 25)	S	R	R	R	R	S	S	R	I	0.5
E. coli (U 26)	S	R	R	S	R	S	R	R	I	0.5
Klebsiella sp. (U22)	S	R	S	S	R	S	I	R	I	0.3
Klebsiella sp. (U23)	S	R	S	S	R	S	I	R	I	0.3
Klebsiella sp. (U29)	S	R	R	R	R	R	R	R	S	0.7
S. aureus (U20)	S	R	S	I	S	S	I	S	S	0.1
S. aureus (U18)	S	R	R	R	R	R	S	S	R	0.7
S. aureus (U21)	S	R	S	I	S	S	I	S	R	0.2
S. aureus (U15)	R	R	S	I	S	S	S	S	S	0.2
P. aeruginosa (U25)	S	R	S	S	R	S	S	S	R	0.3
P. aeruginosa (HVS 06)	S	R	S	S	R	S	S	S	R	0.3

KEY: S: Sensitive, R: Resistant, I: Intermediate and MAR; Multiple antibiotic resistance, Ciprofloxacin (CIP), Cloxacillin (OB), Ofloxacin (OFX), Cefuroxime (CRX), Erythromycin (ERY), Caftazidime (CAZ), Gentamicin (CN), Ceftriaxone (CTR) and Amoxcillin Clavulanate (AM).

CONCLUSION

This study documented the presence of individuals with potentially relevant clinical asymptomatic bacteriuria. The occurrence of female cases of asymptomatic drug resistant bacteriuria within the relatively small population sub set should serve as public health alarm which should be reported to the relevant health care authorities within the school environment. These authorities and other stakeholders should as a matter of urgency commence appropriate enlightenment program targeted at the general female student populations on the need to maintain high personal hygiene standards and avoiding risky behavioral attitudes such deliberate abuse of over-the-counter antibiotics, engaging in unprotected sex and having multiple sexual partners.

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