Plasmid Profile of Bacterial Isolates from Asymptomatic Bacteriuria among Undergraduate Students of a Tertiary Institution in Benin city, Nigeria

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Abstract: Personal lifestyle predisposes individuals to asymptomatic bacteriuria (ASB) whereas uncontrolled spread of antibiotic resistance plasmids among the implicated isolates possibly will hamper effective medical treatment. In this study, a total of fifty (50) urine samples (25 males and 25 females) were obtained from apparently healthy undergraduate students of University of Benin, Benin City, Edo state, Nigeria between September – November, 2018. Prevalence of ASB in the urine samples was determined and the implicated bacterial isolates were identified using standard microbiological methods. Antimicrobial susceptibility testing of the bacterial isolates were carried out using Kirby-Bauer disc diffusion technique. Results obtained revealed that 12 % (males) and 28 % (females) showed ASB. The bacterial isolates and their percentage frequency of occurrence were *Escherichia coli* (60 %), *Proteus mirabilis* (20 %), *Pseudomonas aeruginosa* (10 %) and *Staphylococcus aureus* (10 %). Antibiotics susceptibility tests revealed that ampicillin and augmentin showed 100 % resistance while each of the fluoroquinolones (Ciprofloxacin, Ofloxacin, and Nitrofurantoin) showed 70 % susceptibility being the highest. Considering ASB from the study population, multidrug resistant bacterial isolates which had plasmids constitute 50 % and 33 % of the isolates obtained from 67 % males and 43 % females, respectively. Based on our findings, we recommend implementation of stricter regulations in agricultural, environmental and medical applications of antibiotics especially in developing countries such as Nigeria to reduce spread of antibiotic resistant bacteria implicated in asymptomatic bacteriuria and urinary tract infections.

Keywords: Asymptomatic bacteriuria, plasmid profiling, undergraduate students, prevalence.

INTRODUCTION

The presence of bacteria in human urine which is not as a result of contamination during sample collection is referred as bacteriuria (Colgan *et al*., 2006). According to Imade *et al.,* (2010) and Nicolle (2014), 'asymptomatic bacteriuria' describes a condition where actively multiplying bacteria up to $\geq 10^5$cfu/ml is detected in urine sample of an individual that shows no symptoms of urinary tract infection (UTI). Mostly females rather than males are affected by UTI because of shortened urethra. Among the men, it is prevalent in those having urologic structural abnormalities as well as the aged (Sobel and Kaye, 2014).

Urinary tract infection is a medical condition that establishes the presence and multiplication of bacteria in the urinary tract (Omonigho *et al.,* 2001; Najar *et al.,* 2009). It is grouped into asymptomatic and symptomatic cases based on pathogenesis of the infection (Ojo *et al.,* 2004). The most commonly isolated causative bacterium of UTI from urine sample is *Escherichia coli*. Other bacteria implicated are Enterobacteriaceae, *Pseudomonas aeruginosa, Enterococcus* sp., and group B *Streptococci* (Ezeome *et al.,* 2006; Ezeh *et al*., 2017). Trimethoprim used alone or trimethoprim-sufamethoxazole (SXT) is commonly used for treatment of *Escherichia coli* UTI. In the last decade, resistance of *E. coli* to this drug has increased significantly in the United States of America (Nsofor *et al*., 2016).

Globally, antibiotic resistance to common pathogens is on the increase and worrisome. Several factors attributed to be responsible are human antimicrobial mis-/over-use, animal antimicrobial mis-/over-use, environmental contamination (during the process of manufacture, sewage and disposal

of antibiotics), healthcare transmission, sub-optimal rapid diagnostics (insufficient availability of quick and accurate tests for diagnosis of infections especially in developing countries), sub-optimal preventative medicine/vaccination (dearth of effective vaccines and penchant to accept existing ones), sub-optimal dosing which involves the use of substandard and forged medications by poorly trained community health workers in developing countries, spread of resistant bugs by human travel and mass administration of drugs by an entire country not minding if citizens are sick or not (Castro-Sánchez *et al.,* 2016). Plasmid mediated resistance is the leading cause of antibiotic resistance among many bacteria strains (Ogle *et al.,* 1987). This could be attributed to over dependence and misuse of antimicrobials which increased tremendously in the last five decades (McDermott *et al*., 2003). The production of enzymes that alter the structure of antibiotics, modification of the antibiotics, bypassing certain pathways or acquisition of resistant plasmids from other bacterial strains could play significant role(s) in antimicrobial resistance (Olayinka *et al*., 2009). In 2012, a surveillance of resistance rate of *Escherichia coli* from urine samples of female outpatients in USA to nitrofurantoin, ciprofloxacin, and trimethoprim/sulfamethoxazole was reported to be 0.9 %, 11.8 %, and 22.2 %, respectively (Sanchez, 2016). Bacterial resistance to antibiotics is aided by plasmids or chromosomes (Ogle *et al.,* 1987; Silver, 2003). Therefore, local antibiotic susceptibility pattern of *E. coli* is usually incorporated into clinical decision processes to reduce its prevalence.

Many female students among university population are sexually active. Normal flora of the gut which ascends to the urethra during sexual intercourse or wiping towards the urethra after using the restroom are predisposing factors that could result to UTI (Harvey and Zieve, 2013, Nicolle, 2014). Several studies on prevalence of asymptomatic bacteriuria among undergraduate students in various higher institutions in Nigeria which also includes antibiotic susceptibility testing have been reported (Ayoade *et al*., 2013; Nsofor *et al*., 2016; Ezeh *et al.,* 2017). However, limited studies have been carried out on plasmid profiling of multidrug resistant bacterial isolates implicated in asymptomatic bacteriuria. Therefore, this study seeks to determine the prevalence of asymptomatic bacteriuria among undergraduate students in a tertiary institution in Benin City and also carry out plasmid DNA profiling of the multidrug resistant bacteria in order to provide information that could be useful in modification of newer antibiotics for more effective treatment.

MATERIALS AND METHODS

Study Area/population

The study population comprise fifty (twenty-five males and twenty-five female) apparently healthy undergraduate students of University of Benin, Benin City, Edo state, Nigeria aged between 15 to 30 years who admitted they were not experiencing symptoms of urinary tract infection or undergoing antibiotics treatment at the time of sample collection**.**

Sample collection

Randomized method of sample collection was adopted. A freshly voided urine samples were collected using universal containers containing 0.2 g boric acid crystals between July-August, 2018 from fifty (50) apparently healthy undergraduate students of University of Benin, Benin City, Edo state, Nigeria. All specimens were kept at 4 ^{o}C and processed within 8 hours of collection. Laboratory analysis of the urine samples was carried out at Lahor Research Laboratory and Medical Centre, Benin City, Edo State.

Ethical approval

Ethic approval was obtained from University of Benin, Benin City, Nigeria before commencement of sample collection and subsequent laboratory analysis. Necessary routine pre-sampling explanations prior to securing the subjects understanding and consent was carried out followed by collection of urine samples from the study

population in line with approved ethics for this study.

Microbiological analysis

The urine samples were mixed by gentle inversion and inoculated on blood and MacConkey agar plates which were aerobically incubated at 37 ^{o}C for 24 h and morphological characteristics of the isolates were noted. Pure cultures were obtained by subculturing in fresh agar plates. Gram staining, motility test and biochemical tests on the isolates were carried out (Cheesbrough, 2000). A slightly modified procedure described by Kunin and Buesching (2000) for urine culture using filter paper as a solid-phase dilution device was adopted. Whatman No. 1 filter paper which had absorbing capacity of 0.11 ml was cut to 1 cm by 3 cm and sterilized at 121^{o}C for 15 mins. The sterilized filter paper was picked using a sterilized pair of forceps and dipped into each urine sample and allowed to drain before it was placed on cysteine lactose electrolyte deficiency (CLED) agar plate and incubated overnight at 37 ^{o}C. The number of bacterial colonies on each agar plate was counted after overnight incubation.

$$\text{Bacterial count (cfu/ml)} = \frac{\text{Number of colony count/ml}}{\text{Absorbing capacity of filter paper}}$$

Antimicrobial susceptibility testing

Antibiotic susceptibility testing of the pure isolates was carried out using Kirby-Bauer disc diffusion technique which was adopted from Elenwo *et al*. (2019) in accordance with National Committee for Clinical Laboratory Standards (NCCLS) guideline. Four classes of antibiotics (Oxoid) namely Penicillin- ampicillin (5µg) and augmentin (30 µg), Aminoglycoside - gentamycin (30 µg), Cephalosporins - ceftazidime (30 µg), cefuroxime (30 µg) and ceftriaxone (30 µg), Fluoroquinolones- ciprofloxacin (5 µg), ofloxacin (5 µg) and nitrofurantoin (300 µg) and Macrolides- erythromycin (10 µg) were selected for antimicrobial susceptibility testing of the bacterial isolates. Colonies were suspended into sterile normal saline and the inocula density was adjusted to 0.5 McFarland turbidity standards. A sterile cotton wool swab was inserted into each test tube containing the standardized inocula suspension, rotated with firm pressure on the inside wall of the test tube to remove excess fluid and then used to swab the surface of a freshly prepared dried Mueller-Hinton agar plate. The antibiotic disc was placed on the surface of the inoculated Muller-Hinton agar plate and incubated at 37 ^{o}C for 24 h. After incubation, diameters of zones of inhibition were measured to the nearest millimeter using a transparent meter rule. Duplicate measurement of diameter of zone of inhibition of each clinical isolate was carried out and the mean was calculated. The diameter of zones of inhibition of the clinical isolates were interpreted as susceptible or resistant using CLSI (2009) guideline.

Molecular analysis

The multidrug resistant (MDR) criteria selection were based on bacteria that were resistant to three or more different classes of antimicrobial agents (Magiorakos *et al.,* 2011). Plasmid DNA isolation and profiling was carried out using the method described by Sambrok *et al*. (2001) and Ranjbar *et al*. (2007). Plasmid isolation was carried out on each of the multidrug resistant bacteria using a commercial plasmid isolation kit (ZR Plasmid Miniprep^{TM}- Classic Catalogue number D4O15, D4016 and D4O54) following manufacturer's instructions. Exactly 0.5 ml overnight broth culture of each isolate was centrifuged 10,000×g for 2 min and supernatant was discarded. Two hundred microlitres (200 µL) P1 buffer was added to the pelleted cells followed by 200 µL P2 buffer and thoroughly mixed. The mixture was incubated at room temperature (28±2 ^{o}C) for 2 min. Four hundred microlitres (400 µL) P3 buffer was then added and mixed. The mixture was centrifuged at 16,000×g for 2 min.

The supernatant was loaded inside Zymo-spin™ IIN column and was centrifuged for 30 sec. The flow-through was discarded. Two hundred microlitres (200 µL) endo-wash buffer was added to the column in a collection tube and centrifuged for 30 sec followed by addition of 400 µL plasmid wash buffer and then centrifuged for 1 min. The spin column was placed in a new micocentrifuge tube and 30 µL DNA elution buffer was added before being centrifuged for 30 sec and plasmid DNA pellets was obtained.

Preparation of 0.8 % agarose gel for plasmid DNA detection

The procedure described by Lee *et al.* (2012) with slight modification was adopted. The process involved dissolving 0.8 g agar in 100 ml TrisEDTA buffer and the mixture was heated in a microwave for 5 min to dissolve completely. The mixture was allowed to cool to 56 ^{o}C and 6 µl ethidium bromide was added. The agarose gel was poured inside the electrophoresis chamber with gel comb, and allowed to solidify.

Electrophoresis

Ten microlitres (10 µL) of molecular markers was loaded into the first well. Two microlitres (2 µL) of the loading dye mixed with 8 µl plasmid DNA extract were loaded in the other wells. Electrophoresis was performed at 90 V for 60 min. After electrophoresis, the products were visualized by Wealtec Dolphin Doc UV transilluminator and photographed. Molecular weights were estimated using molecular weight standard of the maker.

Plasmid Curing

Isolates that were positive for plasmid genes were subjected to standard plasmid curing method. An aliquot of overnight culture was inoculated into 9 ml freshly prepared nutrient broth and incubated for 4 h for minimal growth of microorganisms. Then, 1 ml sodium deodecylsulphate (SDS) curing agent was added sufficiently to the mixture to bring the concentration to 1 % and then incubated for 24 h at 37 ^{o}C. One milliliter (1 ml) of cured culture was added to 9 ml of freshly prepared nutrient broth and incubated for another 24 h at 37 ^{o}C. Post plasmid antimicrobial susceptibility testing was carried out.

Statistical analysis

Statistical analysis of data was performed using paired sample T test with the aid of IBM SPSS Statistics software version 20.

RESULTS

Table 1 shows the prevalence of asymptomatic bacteriuria among undergraduate students in a tertiary institution in Benin City, Edo State. In Table 2, the biochemical characteristics of the isolates were listed while Table 3 depicts their frequency of occurrence. Antibiotics susceptibility pattern of bacterial isolates > 10^5cfu/ml in urine samples is presented in Table 4. Depicted in Table 5 was the prevalence of multidrug resistant (MDR) bacterial isolates. Table 6 shows the plasmid profile of multidrug resistant (MDR) bacterial isolates while Table 7 shows the plasmid curing profile of multidrug resistant bacteria isolates. Finally, plasmid DNA profiles of clinical multidrug resistant isolates are shown in Figure 1.

Table 1. Prevalence of asymptomatic bacteriuria among the study population

Gender	Population	Number of subject(s) with significant bacteriuria≥ 10^5cfu/ml	Number of subject(s) without significant bacteriuria≤ 10^5cfu/ml	Number of subject(s) without bacterial growth
Male	25	3 (12 %)	9 (36 %)	13 (52 %)
Female	25	7 (28 %)	11 (44 %)	7 (28 %)
Total	50	10 (20 %)	20 (40 %)	20 (40 %)

Table 2. Biochemical characterization of bacterial isolates from urine samples

Probable Organism	GR	Ox	Cat	Coag	Citrate	Indole	Lactose	H_2S	Motility	Urea
Escherichia coli	-	-	+	-	-	+	+	-	+	-
Proteus mirabilis	-	-	+	-	+	-	-	+	+	+
Pseudomonas aeruginosa	-	+	+	-	+	-	-	-	+	-
Staphylococcus aureus	+	-	+	+	+	-	+	-	-	+

Key: + positive result; - negative result; GR = Gram reaction; Ox = Oxidase, Cat = Catalase; Coag = Coagulase

Table 3. Frequency of occurrence of bacterial isolates from urine samples

Bacterial isolate	Frequency of occurrence	% Frequency (n = 100)
Escherichia coli	6	60
Proteus mirabilis	2	20
Pseudomonas aeruginosa	1	10
Staphylococcus aureus	1	10
Total	10	100

Table 4. Antibiotics susceptibility pattern of isolates (> 10^5cfu/ml) from urine samples

Type of antibiotic N =10	Resistance N = 10	Susceptible N = 10
Ampicillin	100 %	0 (0 %)
Augmentin	100 %	0 (0 %)
Gentamycin	5 (50 %)	5 (50 %)
Ceftazidime	6 (60 %)	4 (40 %)
Ceftriaxone	7 (70 %)	3 (30 %)
Cefuroxime	7 (70 %)	3 (30 %)
Ciprofloxacin	3 (30 %)	7 (70 %)
Ofloxacin	3 (30 %)	7 (70 %)
Nitrofurantoin	3 (30 %)	7 (70 %)
Erythromycin	8 (80 %)	2 (20 %)

Key: N= number of isolates; number of antibiotics

Table 5. Prevalence of multidrug resistant (MDR) bacterial isolates

Gender	No of Isolates	No with MDR
Male	3	2 (67%)
Female	7	3 (43%)
Total	10	5 (50%)

Table 6. Plasmid profile of multidrug resistant (MDR) bacterial isolates

Gender	No of isolates	No without plasmid (%)	No with plasmid (%)
Male	2	1 (50)	1 (50)
Female	3	2 (67)	1 (33)

Table 7. Plasmid curing profile of multidrug resistant bacterial isolates

Gender	No. with plasmid	No. of cured plasmid (%)
Male	1	1 (100 %)
Female	2	2 (100 %)

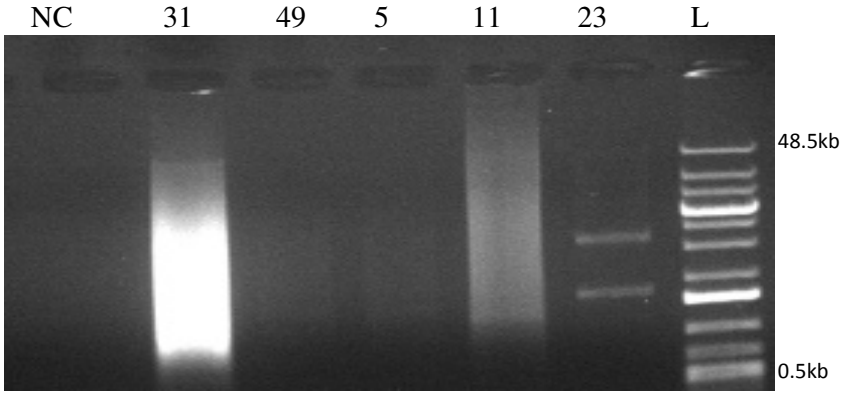

Figure 1: Plasmid DNA profiles of clinical multidrug resistant isolates
Lane (L) is DNA molecular marker (0.5kb-48.5kb ladder) and NC is a no plasmid DNA template (control).
Key: 31-*Escherichia coli*, 49 - *Proteus mirabilis*; 5 - *Proteus mirabilis*; 11- *Escherichia coli;* 23-*E.coli*

DISCUSSION

Prevalence of asymptomatic bacteriuria was observed in 20 % of the entire population used for this study. The females had a higher prevalence (28 %) than the males (12 %). This result was collaborated by Frank-Peterside and Oguike (2006) and Frank-Peterside and Eton (2007) in their separate studies. There was statistical difference (P-value = 0.002) between male undergraduate students and the females which constitute the study population considering number of urine samples with significant bacteriuria, not significant bacteriuria and without bacterial growth. Higher prevalence of asymptomatic bacteriuria in female undergraduate students than the males could be attributed to female undergraduate students within the age group (15-30 years) used for this study were sexually active with multiple sexual partners. Such lifestyle could have predisposed the females to asymptomatic bacteriuria (Olaitan, 2005). Furthermore, the ability of some Enterobacteriaceae species to colonize vaginal introitus increases the risk of young girls to asymptomatic bacteriuria unlike young males. Bacterial species are able to colonize vaginal or any mucosal surface because of its adhesion property (Ezeh *et al*., 2017). According to Olaitan (2005), women are more predisposed to bacteriuria than men because of closeness of female urethra to the anus coupled with its short length which ends beneath the labia. It is rare to report asymptomatic bacteriuria in young adult males which is estimated to be between 5-6 % (Ayoade *et al*., 2013).

Four bacterial isolates namely *Proteus mirabilis, Escherichia coli, Pseudomonas aeruginosa* and *Staphylococcus aureus* were detected in the urine samples. Among the four isolates, *E. coli* which recorded 6 (60 %) frequency of occurrence was most prevalent. The predominance of *E. coli* as the etiologic agent of urinary tract infection (UTI) has been previously reported in separate studies carried out by De Francesco *et al.* (2001) and Al Sweith *et al.* (2005). According to Ayoade *et al.* (2013), high incidence of *E. coli* in numerous cases of UTI could be attributed to already established fact that this bacterium is a commensal of the bowel.

Possible reasons for predominance of *E. coli* might be as a result of poor genital hygienic practices by students who are not used to the habit of proper cleaning of their anus after defecating (front to back) as well as clean their genitals after passing urine (Cheesbrough, 2004). A related study by Nsofor *et al.* (2016) also reported the presence of *Pseudomonas aeruginosa, Staphylococcus aureus* and *Proteus mirabilis* from urine samples of female students categorized as asymptomatic bacteriuria patients. *S. aureus* has been identified as a normal flora of the female perineum and vulva which could explain its presence in the urine samples (Ogefere and Oluka, 2013).It is debatable whether *Proteus mirabilis* is a pathogen, commensal or transient microorganism since it is mainly isolated from the gastrointestinal tract (GIT). Meanwhile, it is believed that *Proteus mirabilis* ascend from GIT to the urinary tract where it cause infection alongside few other bacterial genera. *Pseudomonas* spp. are ubiquitous free-living bacteria which mainly inhabit the soil, seawater, sewage and certain plants. In fact, they easily adapt to any environment. *Pseudomonas aeruginosa* is an opportunistic and nosocomial pathogen frequently isolated from clinical specimen of patients showing signs of urinary tract infections (Su *et al*., 2018).

Antibiotic susceptibility testing of bacterial isolates in the urine samples that had bacteria count above 1 x 10^5cfu/ml revealed that ciprofloxacin, nitrofurantoin and ofloxacin belonging to class of antibiotics known as fluoroquinolones were most effective against Gram negative and Gram positive bacterial isolates. According to Sanchez *et al*. (2016), fluoroquinolones which include ciprofloxacin and levofloxacin could be used for the treatment of uncomplicated urinary tract infection (UTI). This is in agreement with our results which showed that ciprofloxacin demonstrated 70 % susceptibility to bacterial isolates implicated in asymptomatic bacteriuria. Ofloxacin and ciprofloxacin are members of the quinolones known for their effectiveness against a wide range of organisms (Al Sweith *et al.*, 2005). However, the bacterial isolates showed high resistance to Ceftazidime (60 %), Ceftriaxone (70 %) Cefuroxime (70 %), Erythromycin (80 %), Ampicillin (100 %) and augmentin (100 %). This is a strong indication that these antibiotics might not be effective in treatment of UT1. Overuse of the same class of antimicrobials such as third- and fourth-generation cephalosporins and penicillin against commonly isolated bacterial species associated with UTI without carrying out antimicrobial sensitivity testing could be a significant factor which influences the emergence of new bacterial strains highly resistant to different antimicrobials predominantly used for medical treatment (Lemu *et al.,* 2012). Combination of other factors listed by Castro-Sánchez *et al.* (2016) could also be responsible for antibiotic resistance of the clinical isolates against the antibiotics tested. The prevalence of multidrug resistance (MDR) among the bacterial isolates from the population used for this study was 5(50 %). Higher prevalence of MDR from the urine samples of male undergraduate students reported as 2 (67%) was higher than 3 (43 %) for female students. This could be attributed to poor adherence to antibiotic policy, excessive and indiscriminate use of broad-spectrum antibiotics by adventurous male undergraduate students unlike the females (Garba *et al.,* 2012; Akoachere *et al*., 2014). High antibiotic resistance demonstrated by the bacterial isolates against cephalosporins and penicillins could also be attributed to cephalosporinases and penicillinases, respectively. Previous studies reported that urine pathogens are highly resistant to cephalosporins and penicillins. High-level resistance of *Proteus* spp. to Cefotaxime and Ceftazidime has been attributed to acquired cephalosporinases. Plasmid-mediated cephalosprinases is prevalent among Enterobacterales (Girlich *et al*., 2020). Certain strains of *E. coli* expresses penicillinases, cephalosporinases and extended spectrum beta-lactamase (ESBL) responsible for resistance of this

bacterium to antibiotics such as third-generation cephalosporins (Sagna *et al.,* 2019). Although third-generation (Ceftazidime) and fourth-generation (Cefepime) cephalosporins are used in treatment of *Pseudomonas aeruginosa*, through gene mutation this bacterium can acquire resistance which will result in over expression of AmpC β-lactmase (Breijyeh *et al*., 2020). Studies have shown that plasmids of different strains of *Staphylococcus aureus* carry wide variety of multi-drug resistant genes which could be responsible for spread of antimicrobial resistance (Onanuga and Awhowho, 2012).

Bacterial isolates from urine sample of the female undergraduate students contained more plasmids 2 (67%) than that of male students 1 (50 %). It is suggested that self-medication, misdiagnosed non-infectious diseases, bad habit of seeking medical solutions from unqualified drug sellers and acute shortage of well-trained community health workers in most developing countries e.g. Nigeria are combined factors which might have promoted acquisition of multidrug resistance plasmids by some bacterial isolates associated with UTI (Enabulele *et al.,* 2006). Our study shows that all plasmid borne multidrug resistant bacteria isolates from both genders were cured (100 %) of their plasmid by means of treatment with sodium deodecyl sulphate. As a result of the treatment, the isolates became susceptible to antibiotics which they were once resistant having lost their resistant markers. The importance of plasmid curing in the investigation of etiologic agent of diseases as a chemotherapeutic approach cannot be overemphasized as it has improved the policy of antibiotics administration in chemotherapy (Girma *et al.,* 2013).

CONCLUSION

The prevalence of asymptomatic bacteriuria in female undergraduate students were higher than the males. Based on our results, flouroquinolones (ciprofloxacin, ofloxacin and nitrofurantoin) were the most effective antibiotics against bacterial isolates implicated in asymptomatic bacteriuria. On the contrary, penicillins (augmentin and ampicillin) were least effective.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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