MOLECULAR DETECTION *ctx-M*, *TEM and VIM* in ESBL-producing *E. coli* strains isolated from pregnant women in Osogbo.

Abstract

Urinary tract infection (UTI) is a major bacterial infection causing serious health problem in pregnant women. Asymptomatic bacteriuria in pregnancy is associated with pyelonephritis, preterm labour and low birth weight infants. The physiological and anatomical changes in pregnancy facilitate urinary tract infection (UTI) during pregnancy.

Aim: The study was designed to characterise the organisms associated with UTI among pregnant women in Osun State.

Study design: A cross-sectional study design was used to collect mid-stream urine samples between March 2018 to September 2018 from 150 pregnant and 50 non-pregnant women which serve as control. Samples were inoculated into Cysteine Lactose Electrolyte Deficient (CLED) medium, subcultured onto MacConkey and Blood agar plates. A standard agar disc diffusion method was used to determine antimicrobial susceptibility pattern of the isolates and the molecular detection of the antibiotic resistant genes were done. Data were subjected to descriptive statistics.

Results: The ages of women enrolled in this study ranges from 22 to 43 years (mean ± standard deviation = 25 ± 4.7 years). The predominant bacteria identified were *E. coli* (46.4%), *S. aureus* (14.3%), coagulase negative Staphylococci [CoNS] (14.3%), *Klebsiella* species (12.4%) and *Enterobacter* species (10.6%). Majority of Gram-negative bacteria isolates were resistant to ampicillin (70%), cefotaxime (62%), while 75–100% of the Gram positive isolates were resistant to ampicillin. Multiple drug resistance was observed, all the *E. coli* isolates were resistant to Cefotaxime, of *E. coli* isolates, 4, 3 and 6 were positive for the VIM, CTX-M and TEM genes.

Conculsion: Similarly, the risk of UTI was higher in those had previous UTI history (OR = 2.29, 95% CI = 1.15–4.56, P = 0.019) as compared to those who had no previous history of UTI.

Keywords: UTI, pregnant women,

Introduction

Urinary tract infection is a common health problem among pregnant women (1). Urinary tract infection (UTI), otherwise known as bladder infection, is a bacterial inflammation in the urinary tract. Pregnant women are at increased risk for UTI's especially during first and

second trimesters and are caused by bacteria, fungi and some viruses. It has been observed that pregnant women have a propensity to develop recurrent UTIs (2). Overall, UTI can be dangerous for both the mother and fetus. Complications that can arise include preterm delivery, premature rupture of membrane and increased incidence of intrauterine growth restriction. Also, preeclampsia, caesarean delivery, anemia, sepsis, and septic shock may also be associated with UTI in these patients (3). The rate of E. coli infection among women is alarming, thereby posing a very big health issue among pregnant and non-pregnant women.

E. coli is the most incriminating pathogen causing UTI. Lack of proper diagnosis can lead to serious clinical problems and outcomes for women, especially pregnant women.

Drug resistance in *E. coli* must be promptly addressed before multiple resistant strains start emerging and spreading in the various communities. Therefore the study is aimed at characterizing molecularly *Esherichia coli* among pregnant and non-pregnant women in Osun State.

Materials and Method

Study area

The study was conducted in the city of Osogbo, Osun State from March 2018 to September 2018.

Ethics Statement

This work was performed according to University ethics committee code of conduct, verbal informed consent was obtained from all participating subjects.

Study population

The study population were pregnant women attending antenatal clinic (ANC) at Onward specialist hospital, Agunbelewo, Osogbo and Primary health centre, Atelewo, Osogbo during the study period, and some selected non-pregnant women around Osogbo metropolis, those who did not initiate antimicrobial drug therapy for at least 2 weeks prior to sample collection.They were registered in the project register where basic demographic data about the patients including name, age, sex, ward etc. were recorded.

Sample size calculation was done using Leslie Fisher's formula

Sampling methods

Study participants were selected using simple random sampling technique for the selection of pregnant and the non-pregnant women across Osogbo town.

The calculated sample size was proportionally distributed to Onward hospital (n = 54), Primary Health Centre, Atelewo (n = 96), and non pregnant women (n= 50).

Sample collection

A total of 200 mid-stream urine (MSU) samples were collected from the participants. Ten to fifteen milliliter of freshly voided midstream urine samples were used for microscopic investigation and culture media inoculation. Urine samples were processed within 4 h of collection (4).

In the laboratory, urine samples were centrifuged at 1500 RPM for 5 min. After centrifugation a drop of the sediment was placed on the grease free slide, covered with cover slip and examined under the microscope using the high power objective lens (40X).

Reporting system for microscopic identification was done for pus cells, red blood cells (RBCs), epithelial cells, casts, crystals, yeast cells (5).

Isolation and identification

Standard loop technique was used to place 0.001 ml of urine for inoculation on Cysteine lactose electrolyte deficient (CLED) medium, Blood agar, MacConkey agar and incubated at 37 °C for 24 h (6). The numbers of colonies were counted to quantify organisms.

Diagnosis of UTI is defined on the basis of significant colony count of ≥105 cfu/ml for Gram-negative and Gram-positive bacteria (7).

Growths on the culture media were identified by using bacterial growth characteristics (morphology), Gram staining and general biochemical tests (8).

Antimicrobial susceptibility testing (AST) of uropathogens.

The antimicrobial susceptibility testing of all isolates was done using commercial disks following the standard disk diffusion method recommended by the National Committee for Clinical Laboratory Standards (NCCLS 2012).

The drugs that tested were Penicillin (PEN, 30 µg), Ampicillin (AMP, 30 µg), Ciprofloxacin (CPR, 5 µg), Levofloxacin (LEV, 10 µg), Cefuroxime (CPX, 10 µg), Cefotaxime (CTX, 10 µg), Tetrecycline (TET, 300 µg) and Meropenem (MEM, 1.25 µg).

All the antimicrobials used for the study were purchased from Oxoid Limited Bashing store, USA.

Genotypic Identification of *E. coli* Isolates

E. coli isolates were grown overnight at 37°C on blood agar or for 24 h on LB agar without salt for infection or biofilm assays, respectively.

DNA Extraction

The DNA molecules of the isolates were extracted by suspending bacterial colonies in 500 µl of sterile distilled water in appropriately labelled Eppendorf tubes.

The cells were washed three times in sterile distilled water while vortexing and centrifuging at 10, 000 rpm. Tubes were covered and sealed with paraffin tape to prevent accidental opening.

After the last washing, the bacterial suspensions were boiled at 100 °C for 10 minutes in water bath and cold shocked in ice for 2 minutes. The boiled suspension contained the DNA.

PCR Amplification

The polymerase chain reaction was set up in a PCR vial, after adding the master mix, the forward and reverse primers and the extracted DNA. A 20µl reaction containing 2µl of 10X buffer, 1µl $Mgcl_2$, 0.8µl dNTPs, 0.5µl of forward primer, 0.5µl of reverse primer, 0.2 µl Taq polymerase, 10µl of nuclease free water and 5µl of DNA lysate was used for PCR.

Amplification was subjected to initial denaturation at 95C °for 5min, followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 60°C, 56°C, 54°C, 47°C, 52C° for 1 min, for *ctx-M,* VIM and TEM respectively, extension at 72°C for 1 min and final extension procedure was carried out at 72°C for 10min.

Gel Electrophoresis

At the completion of the amplification, PCR products were resolved on l.5% agarose gel prepared by dissolving 1.5g of agarose powder in 100 ml of 1X Tris-borate-EDTA (TBE) buffer solution inside a clean conical flask.

The 1.5% agarose solution was heated in a microwave oven for 2-3 minutes and was observed for clarity which was an indication of complete dissolution. The mixture was then allowed to cool to about 50 °C after which 0.5 µl of ethidium bromide was then added. It was allowed to cool further and then poured into a tray sealed at both ends with support to form a mould with special combs placed in it to create wells.

The comb was carefully removed after the gel had set and the plate was placed inside the electrophoresis tank which contained 1X TBE solution stained with 1ug/ml of ethidium bromide solution and loaded to the well of the agarose gel. The power supply was adjusted to 100 volts for 25

minutes. For each run, a 100 base-pair molecule weight DNA standard (size marker) was used to determine the size of each PCR product.

The DNA bands were then visualized with a short wave ultraviolet trans-illuminator and photographed using gene gel bio imaging system. The PCR product was then analyzed.

Statistical analysis

Data from laboratory investigation and questionnaire survey was entered into Microsoft Excel Spreadsheet. Descriptive statistics was used to summarize the data.

Chi-square test was used to assess differences in the proportions of culture positive and negative participants. The prevalence of UTI was calculated. To determine predictors of bacteriuria, odds ratios were calculated using likelihood estimation technique.

Independent variables (age, level of education, monthly income, parity, residence, washing habit and previous history of UTI) which are non-collinear and with P-values ≤ 0.25 in univariable logistic regression analysis were further tested via multivariable logistic regression in order to get adjusted odds ratios and significant predictors of UTI in pregnant women. P-value of <0.05 was considered statistically significant.

RESULTS

There were 200 women enrolled in this study; 150 pregnant women, and 50 non-pregnant women (Table 1). The age of pregnant women enrolled in this study ranges from 22 to 43 years with a mean age of 33 years (Standard Deviation [SD] = 4.7), and non-pregnant women from 25-41 years, with a mean age of 32 years (Table 3).

From 200 urine samples, 29 (14.5%) (95% CI: 14.4–23.54%) were culture positive with colony count of more than 10^5 cfu/ml. Of the culture positive urine samples, 21 (72.0%) and 8 (28.0%), were Gram-negative and Gram-positive bacteria, respectively. Five bacterial species of UTI were isolated in which *E. coli* (n = 10) was the predominant bacteria followed by *S. aureus* and coagulase negative *Staphylococci* [CoNS] (n = 8) (Table 4). Microscopic examination of urine samples indicated the presence of pus cells in 35 (17.5%), and epithelial cells in 168 (84%) of samples examined.

Table 1: Positive samples of pregnant and non-pregnant women.

Subjects	No of Samples	Positive culture	Percentage
Pregnant women	150	21	57.14
Non-pregnant women	50	8	42.86

Table 2: Gestational ages of pregnant women enrolled in this study.

Hospital	Patient age range (yr)	Gestational age range (wk)	Total (%)	First trimester	Second trimester	Third trimester
Onward	25-42	10-40	54 (27%)	14	21	9
Atelewo	22-43	8-39	96 (48%)	31	49	16

Table 3: Distribution of the Uropathogens among pregnant and non-pregnant women.

Uropathogen	Distribution			
	Onward	Atelewo	Nonpregnant	Total
Escherichia coli	3	5	2	10
Staphylococcus aureus	0	1	2	3
Staphylococcus saprophyticus	2	2	1	5
Enterobacter	0	2	0	2

aerogenes				
Klebsiella pneumoniae	2	4	3	9
Total	7 (25%)	14 (49%)	8 (26%)	29

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170 Table 4: Antibiotic susceptibility pattern of the *E. coli* isolates.

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Isolates	PEN	AMC	CTX	CPX	TET	LEV	MEM	CIP
Onward	R	R	S	S	R	S	R	S
	S	R	S	S	R	S	R	S
Atelewo	S	R	R	S	R	S	R	S
	R	S	R	S	R	S	R	R
	S	S	S	R	R	S	R	S
	S	R	R	R	S	S	R	R
	S	R	R	R	S	S	R	S
Nonpregnant	R	R	R	R	R	S	R	S
	R	S	R	R	S	S	R	S
	S	R	S	R	S	S	R	R

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173 Key: PEN- Penicillin, AMC- Ampicillin, CTX- Cefotaxime, CPX- Cefuroxime, CIP- Ciprofloxacin,
174 TET- Tetrecycline, LEV- Levofloxacin, MEM- Meropenem

175 Table 5: Table showing the primers used in the PCR Amplification process.

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Primer	Sequence 5^1 -3^1	Base pair (bp)	Annealing temp. (ºC)
CTX-M F CTX-M R	CGATGTGCAGTACCAGTAA TTAGTGACCAGAATAAGCGG	585	60
TEM F TEM R	CCCCGAAGAACGTTTTC ATCAGCAATAAACCAGC	517	52
VIM2004A	GTTTGGTCGCATATCGCAAC	390	54

VIM2004A	AATGCGCAGCACCAGGATAG		

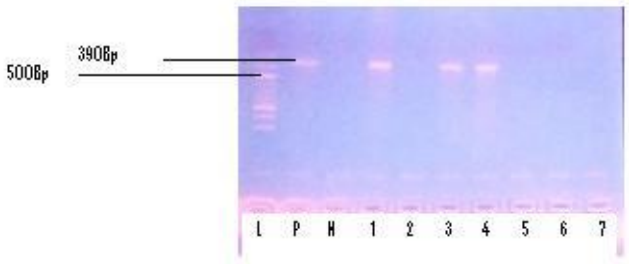


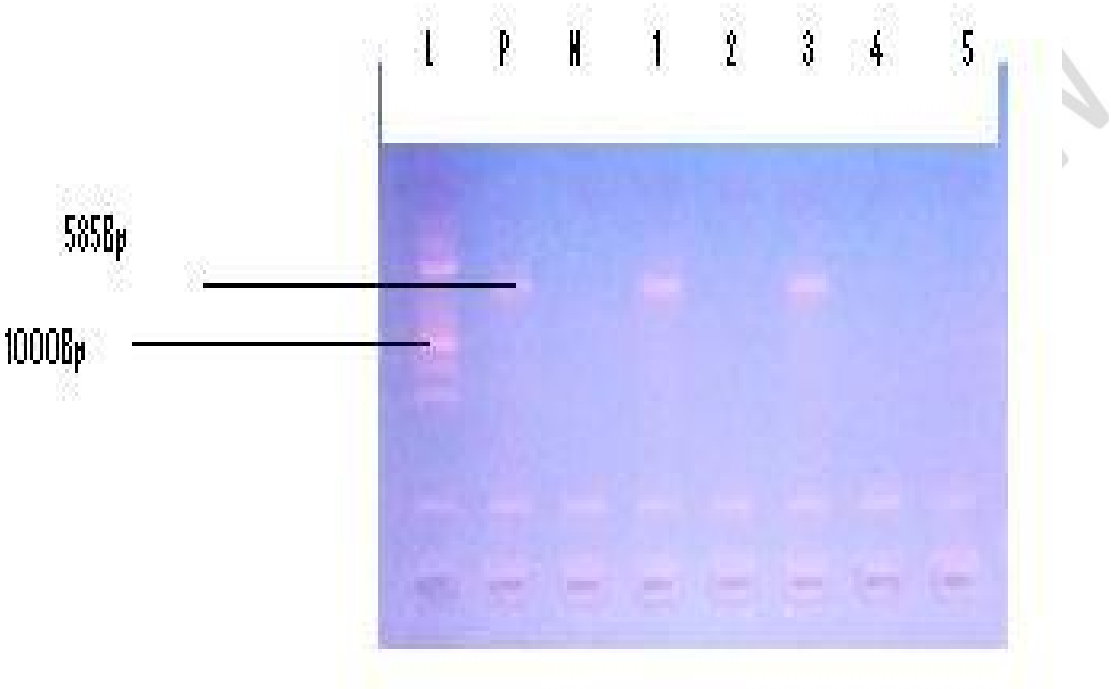
Figure 1: Agarose gel electrophoretogram of (VIM) *Escherichia coli* after PCR analysis *Escherichia coli* isolates which bands at 390 bp

Key:

L (100 bp ladder)

P –Positive

N-Negative



205 Figure 2: Agarose gel electrophoretogram of CTX-M–type β-lactamases (CTX-Ms)
206 *Escherichia coli* after PCR analysis. *Escherichia coli* isolates which bands at 585 bp

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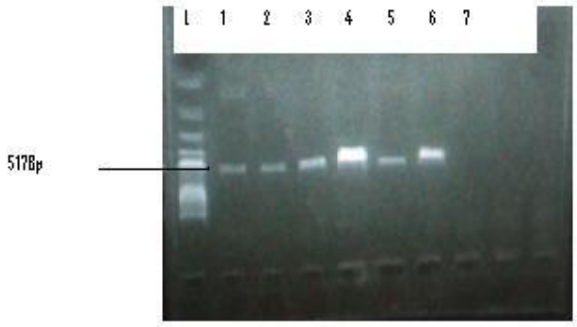

Figure 3: Agarose gel electrophoretogram of TEM *Escherichia coli* after PCR analysis which bands at 517 bp

Results and Discussion

Results

There were 200 women enrolled in this study; 150 pregnant women, and 50 non-pregnant women (Table 1). The age of pregnant women enrolled in this study ranges from 22 to 43 years with a mean age of 33 years (Standard Deviation [SD] = 4.7), and non-pregnant women from 25-41 years, with a mean age of 32 years (Table 3). From 200 urine samples, 29 (14.5%) (95% CI: 14.4–23.54%) were culture positive with colony count of more than 10^5 cfu/ml. Of the culture positive urine samples, 21 (72.0%) and 8 (28.0%), were Gram-negative and Gram-positive bacteria, respectively. The most predominant isolate was *E. coli* 10 (47.6% of the Gram-negatives, 34.5% of all isolate). Microscopic examination of urine samples indicated the presence of pus cells in 35 (17.5%), and epithelial cells in 168 (84%) of samples examined.

Five bacterial species of UTI were isolated in which *E. coli* was the most prevalent organism isolated from women with significant bateriuria and was isolated from 10 cases (52.78%) followed by *Klebsiella* spp. in 9 cases (25%), *S. aureus* in 3 patients (13.9%), *S. saprophyticus* was isolated in 5 cases (5.56%) and *Enterobacter aerogenes* in two patients

(2.78%). (Table 3). The prevalence of symptomatic and asymptomatic UTI was 20.4% (95% CI: 13.09–29.46%) and 17.8% (95% CI: 12.70–23.83%) respectively. Of the 29 bacterial isolates, 10 (47.6%) were from private hospital and the remaining 19 (52.4%) were from periurban government hospital and selected people. Monthly income, personal hygienic habits and previous history of UTI are significantly associated with prevalence of UTI ($P < 0.05$). One hundred and twenty five (62.5%) of study participants had income level of 20000 – 50000 Nigerian naira (21.23–42.37 USD) and seventy five earn above. On the basis of their lifestyle about 115 (57.5%), had a lower level of personal hygiene. About 11 (38%) of positive pregnant women had previous history of UTI.

Antimicrobial susceptibility pattern of bacterial uropathogens

Bacterial uropathogen isolates from patients with UTIs revealed the presence of high levels of single and multiple antimicrobial resistance against commonly prescribed drugs. Gram-negative isolates showed higher resistance pattern in comparison to Gram-positive for most of commonly prescribed antibiotics. *E. coli*, which is the predominant cause of UTI, showed high percentage of resistance to ampicillin and low resistance to ciprofloxacin and penicillin (Table 5). All the E. coli isolates are sensitive to levofloxacin, and all are resistant to meropenem.

Multiple drug resistance patterns of the isolates

Multiple drug resistances (MDR) i.e., resistance to two or more antimicrobial drugs, was found in all the E. coli isolates (100%). All isolates of Gram-negative and Gram-positive bacteria were resistant to at least two antimicrobials. There were no isolates sensitive to all antibiotics tested (Table 5).

Associated risk factors

Univariable logistic regression analysis showed significant association between prevalence of UTI and income level ($P = 0.046$), residential place ($P = 0.029$), personal hygiene ($P = 0.04$) and previous history of UTI ($P = 0.028$). Multivariable logistic regression revealed that the

odds of acquiring UTI in pregnant women is 4.78 times higher than those of non-pregnant women (95% CI of OR = 1.03–22.21, P = 0.046) (Table 4). Similarly, the risk of UTI infection is twice and 2.04 times higher in those who had previous history UTI infection (OR = 2.29, 95% CI of OR = 1.15–4.56, P = 0.019), as compared to those who had no previous history of UTI.

Amplification of the resistant genes

The primers used for this PCR are *ctx-M*, *TEM*, *VIM*, (Table 5), and the process carried out at normal conditions as described earlier. The *ctx-M* resistant genes were observed at 390bp (Fig 1). Resistant genes *TEM*, *VIM*, were observed at 585bp and 517bp (Fig 2) and (Fig 3).

Discussion

The study was undertaken to determine the occurence of urinary tract infection caused by *E. coli* and other uropathogens among pregnant and non-pregnant women and also to analyse the risk factors for predisposition to UTI, and resistance patterns. The culture positive urine samples belong to Gram-negative and Gram-positive bacteria. The low incidence of urinary tract infection reported in the private hospital (Onward) may be attributed to the extensive health care talk given regularly by the staff of the hospital's ante-natal section, higher level of education and exposure, higher standard of living, among others. This study shows a higher incidence of urinary tract infection among pregnant women than non-pregnant women. It is commonly accepted that a high frequency of UTI during pregnancy is due to physiological changes that the human body undergoes in the pregnant condition (10). The higher incidence of urinary tract infections in pregnant women might be as a result of a variety of factors, such as more open and exposed uterus and bladder due to distended stomach (9) and incomplete and in coordinate voiding of urine in pregnant women and encourages infection of the urinary tract (9). A total number of 29 isolates were obtained from the 29 women with positive cultures, that is only one bacterial was isolated from each patient, suggesting a mono-microbial nature of infection in the study population. The pattern and frequency of occurrence of the bacterial isolates found in this study is similar to those reported by other workers. Lavigne *et al*. 2011 reported in their study that *E. coli* was the most commonly isolated pathogen in significant bacteriuria (11). They reported that *E. coli* was responsible for 52% of cases of urinary tract infection, *Klebsiella* spp. (14%), *Proteus* spp. (95%) and

Enterobacter aerogenes in 4%. A higher percentage of the organisms found in this study were isolated mainly from pregnant women. *E. coli* was isolated in 8 of the pregnant women, *Enterobacter aerogenes* in just two, *S.aureus* in one, *S. saprophyticus* in four and *Klebsiella* spp.in four of the pregnant women. The result of this study shows that 100% of the E. coli isolates were sensitive to Levofloxacin, 33.3% to Ampicilin, 55% to penicillin 36% to cefotaxime, 39% to cefuroxime, 77.8% to ciprofloxacin and 0% to meropenem.. The antibiotic sensitivity test of this study shows that Levofloxacin was the most effective antibiotic in *in vitro* testing against *E. coli* isolates followed by ciproflaxin which was effective against 77.8% of the isolates. A reduced sensitivity of *E. coli* to nitrofuratoin was observed in this study as only 45% of the *E. coli* was sensitive to the antibiotics as opposed to the findings of Goldraichi and Manfrori (12), who reported a higher efficacy of the drug against *E. coli in vitro*. They reported a sensitivity of *E. coli* to nitrofuratoin of 92, 95 and 94%, respectively over a three-year period. Olowu and Oyetunji reported a 57.9% sensitivity of pathogens towards nitrofuratoin(13). In this study, Meropenem was the most ineffective antibiotic in *in vitro* testing, since 100% of the pathogens were resistant to it. Resistance of *E. coli* to cefuroxime was 40% and is in contrast to results obtained elsewhere. Christiaen et al. (1998) reported a resistance of 17% to cotrimoxazole and a similar result was reported for resistance to quinolones. This study shows a high level of resistance to cefuroxime, ampicillin and tetracyclin as more than 60% of the isolates were resistant to them *in vitro* and, as such, these antimicrobials may not be suitable for treating case of UTI caused by E. coli in Osogbo. Multiple drug resistance was observed among *E. coli,* of *E. coli* isolates, 4, 3 and 6 were positive for the VIM, CTX-M and TEM genes respectively. Edelstein and colleagues reported that CTX-M beta lactamases have a destructive effect on Cefuroxime.

Conclusion

The predominant bacteria identified were *E. coli*,mMajority of Gram-negative bacteria isolates were resistant to ampicillin , cefotaxime , while Gram positive isolates were resistant to ampicillin. Multiple drug resistance was observed, all the *E. coli* isolates were resistant to Cefotaxime, some of the *E. coli* isolates were positive for the VIM, *ctx-M* and TEM resistant genes.

Recommendation

Health education, continuous and collaborative surveillance of UTI and antimicrobial resistance pattern are essential to reduce the consequence of symptomatic and asymptomatic

bacteriuria and multi-drug resistant bacteria in pregnant women. Enlightenment programs informing the general public on the importance of good personal hygiene and the implications if neglected should be encouraged. This will not only reduce the risk of UTIs but other infections as well. Likewise, there should be continuous education for pregnant women on the need to maintain a high level of personal hygiene during pregnancy as they are at high risk for the infection. They should also be educated on the importance of routine medical check-up during the period of pregnancy.

Conflict of Interest:

There is no conflict of interest to be declared

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