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Antibiotic Susceptibility and Detection of Resistance Genes of *E. coli* among Healthy Pregnant Women in Designated Hospitals around Osogbo, Osun State, Nigeria

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ABSTRACT: Treatment of *E. coli* is now a challenge globally, due to continuous increase in resistance towards commonly prescribed antibiotics, thus posing a threat to treatment. Hence, the aim of the study is to determine antibiotics sensitivity and resistance genes of *E. coli* from apparently healthy pregnant women in Osogbo, Osun State, Nigeria using standard methods. A cross-sectional study design was used to collect 150 mid-stream urine (MSU) samples from apparently healthy pregnant women. Standard inoculating loop technique was used by culturing 0.001 ml of MSU on Cysteine Lactose Electrolyte Deficient (CLED) agar, Blood agar and MacConkey agar and incubated at 37 °C for 24 h. A standard agar disc diffusion method was used to determine antimicrobial susceptibility pattern of the isolates. The molecular detection of resistance genes was done using PCR techniques. The ages of women enrolled in this study ranged from 22 to 42 years (mean ± standard deviation = 31 ± 4.7 years). Eight isolates were positive for *E. coli*. *Escherichia coli* showed high percentage of resistance to ampicillin and low resistance to ciprofloxacin and penicillin. All the *E. coli* isolates were sensitive to levofloxacin, and most were resistant to Meropenem. Multiple drug resistance was observed in all the isolates. Resistance genes in *VIM* 390bp, *bla ctx-M* 585bp and *TEM* 517bp were detected in some of the representative *E. coli* isolates profiled. This study identified the presence of Multi-drug resistance genes in *E. coli* associated UTI among pregnant women in Osogbo.

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Escherichia coli (*E.coli*) is most common Gram negative aerobic bacilli found in gastrointestinal tract causing urinary tract infection (Ade-Ojo *et al*., 2013; Abejew *et al*., 2014). Urinary tract infection (UTI) is common during pregnancy and can be associated with negative outcomes for both the mother and foetus. Increased risk of infection among these subjects has been attributed to physiological changes, and less focus has been placed on *Escherichia coli*, the most frequent causative agent (Thomas and Hooton, 2012, Alfred *et al*., 2013). Urinary tract infection (UTI) is a major bacterial infection causing serious health problem in pregnant women. The physiological and anatomical changes in pregnancy facilitate urinary tract infection (Tadese *et al*., 2014, Dash *et al*., 2013). Moreover, UTI can be dangerous for both the mother and foetus. Complications that can arise include preterm delivery, premature rupture of membrane and increased incidence of intrauterine growth restriction. Treatment of *E. coli* is now a challenge due to continuous increase in resistance towards commonly prescribed antibiotics, thus posing a threat to treatment (Delzell *et al*., 2000, Alemu *et al*., 2012). The rate of *E. coli* infection among women is alarming, thereby posing a very big health issue among women generally (Demilie *et al*., 2014). *Escherichia coli* isolates showed sensitivity to Ofloxacin, Ceftazidime, Augumentin, and Gentamycin but resistance towards Tetracycline, Amoxicillin, and Cotrimoxazole Also, drug resistance in *E. coli* must be promptly addressed before multiple resistant strains start emerging and spreading in the various communities. Hence, the objective of the study is to evaluate antibiotic susceptibility and resistance genes of *E.coli* amongst

apparently healthy pregnant women in designated hospitals around Osogbo, Osun State, Nigeria.

MATERIALS AND METHODS

Study area: The study was carried out in Osogbo in Osun State and the health care facilities that were randomly selected.

Study design: A cross-sectional examination was performed on the collected urine samples from pregnant women (outpatients) in attendance for antenatal clinic (ANC) at onward specialist hospital, Agunbelewo, Osogbo (Private hospital) and Primary health centre, Atelewo, Osogbo (Government hospital). This work was performed according to University ethics committee code of conduct and informed consents were gotten from the subjects. The subjects employed were asymptomatic pregnant women that attended antenatal clinic and did not initiate antimicrobial drug therapy for about two weeks prior sampling. Study participants were selected by simple random technique within the pregnant women attending each clinic. The calculated sample size was proportionally distributed to Onward hospital (n = 54), Primary Health Centre, Atelewo (n = 96).

Methods: A total of 150 mid-stream urine (MSU) samples were collected from pregnant women attending antenatal clinic and nonpregnant women in the study period. 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 (8). 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). 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 (Tadese *et al*., 2014; Alemu *et al*., 2012).

Antimicrobial sensitivity test (AST) of E. coli isolates: Using a sterile wire loop, 3 well-isolated colonies of *E. coli* isolate was touched and emulsified in 4 ml of sterile physiological nutrient broth. In a good light, the turbidity of the suspension was matched to the turbidity standard (MacFarland 0.5). Using a sterile swab, a plate of Mueller Hinton agar was inoculated.

The excess fluid was removed by pressing and rotating the swab against the side of the tube above the level of the suspension. The swab was evenly streaked over the surface of the medium in three directions, rotating the plate approximately 60° to ensure even distribution. With the petri dish lid in place, it was allowed to stay 5 minutes for the agar surface to dry. Using sterile forceps, the antimicrobial discs (Penicillin (PEN, 30 µg), Ampicillin (AMP, 30 µg), Ciprofloxacin (CPR, 5 µg), Levofloxacin (LEV, 10 µg), Cefuroxime (CPX, 10 µg), Cefotaxime (CTX, 10 µg), Tetracycline (TET, 300 µg) and Meropenem (MEM, 1.25 µg)) were placed on the inoculated plate evenly, about 15 mm from the edge of the plate and no closer than 25 mm from disc to disc.

Each disc was lightly pressed down to ensure its contact with the agar. The endpoint of inhibition was defined as where growth started.

Molecular detection of resistant isolates: Extraction of DNA: The DNA molecules of the *E. coli* isolates were extracted using boiling method.

DNA Amplification by PCR: The PCR was prepared in a PCR vial, having added the master mix, forward and reverse primers (Table 1), and also the DNA extracted. A 20 µl reaction containing 2 µl of 10X buffer, 1µl Mgcl2, 0.8µl dNTPs, 0.5µl of forward primer, 0.5 µl of reverse primer, 0.2 µl Taq polymerase,10 µl of nuclease free water and 5 µl of DNA lysate was used for PCR. Amplification was subjected to first denaturation at 95 ^{0}C for 5min, followed by 35 denaturation cycles at 95 ^{0}C for just 1 min, annealing at 60 ^{0}C, 54 ^{0}C, 52 ^{0}C for 1 min, for *CTX-M, VIM*, and *TEM* respectively, extension at 72 ^{0}C for 1 min and final extension procedure was performed at 72 ^{0}C for 10 min.

Table 1: Primers used in the PCR Amplification of antibiotic resistance genes in the *E. coli* isolates

Primer	Sequence 5^1 -3^1	Base pair (bp)	Annealing Temp. (ºC)
CTX-M F	CGATGTGCAGTACCAGTAA	585	60 (Livermore *et al*., 2007)
CTX-M R	TTAGTGACCAGAATAAGCGG		
TEM F	CCCCGAAGAACGTTTTC	517	52 (Mesa *et al*., 2007)
TEM R	ATCAGCAATAAACCAGC		
VIM F	GTTTGGTCGCATATCGCAAC	390	54 (Fischer et al., 2012)
VIM R	AATGCGCAGCACCAGGATAG		

Key: F- Forward, R- Reverse

RESULTS AND DISCUSSION

*Antimicrobial susceptibility pattern of bacterial isolates***:** *E.coli* from pregnant women showed presence of much antimicrobial resistance, both single and multiple against the commonly recommended drugs. *E. coli* had high resistance pattern to ampicillin, but low resistance towards ciprofloxacin and penicillin (Table 2). Antibiotic resistance pattern of the eight isolates of *E. coli* in the three healthcare facilities (Table 3).

Multi-drug resistance among the isolates: Multi-drug resistance (MDR), the majority of the *E. coli* isolates were MDR (resistance to ≥1 agent in ≥3 antibiotic categories) which is resistance to more than two classes of antimicrobial drugs, was present among all *E. coli* isolates (100%). Resistance genes *VIM* bound 390 bp (Fig, 1), *bla ctx-M* bound 585 bp (Fig.2) and *TEM* bound at 517 bp (Fig.3) were detected in some of the representative *E. coli* isolates by gene profile. The study was aimed at determining the rate at which *E. coli* cause urinary tract infection among pregnant women and the resistance patterns. Of all 150 pregnant women which were between 22 and 43 years of age that participated, 8 (5.3 %) were positive for *E. coli.* The low UTI incidence noticed maybe as a result of the extensive health care talk given regularly by the staff of the hospitals ante-natal section, among others. 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. In this study, Meropenem showed the highest ineffective antibiotic ability for in vitro testing, since 100 % (all) of the isolates showed resistance to it. Resistance to cefuroxime in *E. coli* was 50 % and is contrasting to results from other places.

Table 2: Antibiogram of Bacterial Isolate

	Antibiotic Effect on *E. coli* isolates (n %)	
Antimicrobial agent (Conc.)	R (%)	S (%)
Penicillin (30 µg)	3 (37.5 %)	5(62.5 %)
Ampicillin (30 µg)	6 (75 %)	2(25 %)
Cefotaxime (10 µg)	5 (62.5 %)	3(37.5 %)
Cefuroxime (10 µg)	4(50 %)	4(50 %)
Tetracycline (300 µg)	4 (50 %)	4(50 %)
Levofloxacin (10 µg)	0 (0 %)	8(100 %)
Meropenem (1.25 µg)	8 (100%)	0(0 %)
Ciprofloxacin (5 µg)	2 (25 %)	6(65 %)

Key*: R = Resistant, S= Sensitive*

Table 3: Antibiotics resistance Pattern table

Sample Source		**Resistant Antibiotics**					
Onward	Isolate 1	PEN	AMC	TET	MEM		
Specialist	2	AMC	TET	MEM			
Hospital	3	AMC	CTX	TET	MEM		
Primary Health	Isolate 1	PEN	CTX	TET	MEM	CIP	
Centre	2	CPX	TET	MEM			
	3	AMC	CTX	CPX	MEM	CIP	
	4	AMC	CTX	CPX	MEM		
	5	PEN	AMC	CTX	CPX	TET	MEM

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

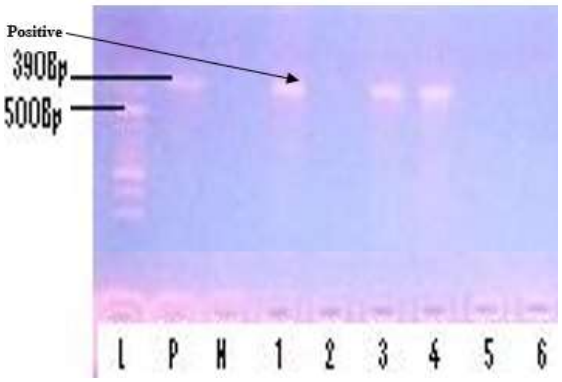

Fig .1: Agarose gel electrophoretogram of (VIM) *Escherichia coli* isolates from pregnant and non-pregnant women.

Key: L=500 bp ladder; P= Positive control; N= Negative control; Positive isolates=1, 3, 4

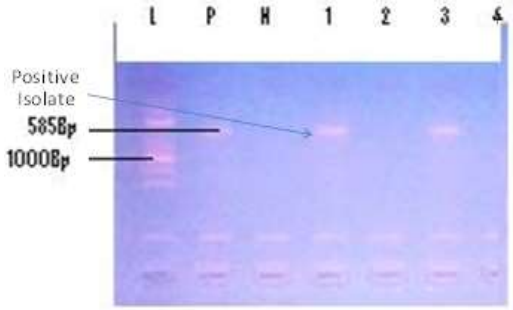

Fig 2: Agarose gel electrophoretogram of *ctx-M*–type β-lactamases *Escherichia coli* isolates from pregnant and non-pregnant women. *Escherichia coli* isolates which bound at 585 bp Key: L= 100 bp ladder; P= Positive isolates; N= Negative isolates

This shows a high resistance level to cefuroxime, ampicillin and tetracycline because over 40 % of all the isolates showed resistance towards them in vitro, and so, those antimicrobials might not be suitable for treating case of UTI of *E. coli* origin in Osogbo. The resistance of the *E. coli* to ampicillin, cefotaxime, and cefuroxime, observed in this study was in agreement with the report of Padilla *et al.* (2010) and Alikhani *et al*. (2014).

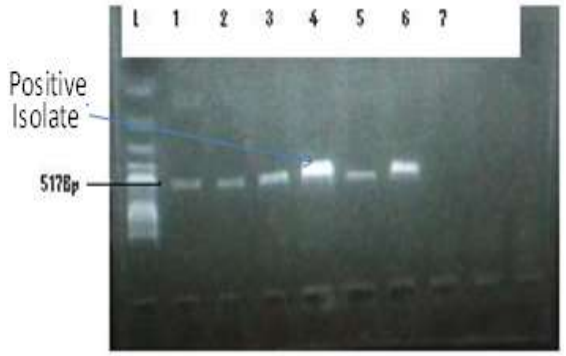

Fig 3: Agarose gel electrophoretogram of TEM *Escherichia coli* isolates
Key: L= Ladder, Positive isolates= 1, 2, 3, 4, 5, 6; Negative isolate= 7

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. 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 (Fischer *et al*., 2012). The occurrence of Multi-drug resistance MDR was observed in all the isolates of the two study centers and this finding is also not different from the study earlier reported by Thakur et al. (Thakur *et al*., 2013). The presence of MDR therefore reduces the number of available therapeutic antibiotics that can be used against *E.coli* (Fred *et al*., 2015; Gebrekirstos *et al*., 2017).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 [Edelstein *et al*., 2003]. The detection of VIM suggested the presence of carbapenem-resistant gene and TEM the production of betalactamases. Extended-spectrum beta lactamase by *E .coli* may destroy the potency of the third generation cephalosporins. ESBLs are plasmid-encoded or chromosomally encoded β-lactamases with broad activity against penicillins and cephalosporins. They function by splitting the amide bond of the β-lactam ring, thus inactivating β-lactam antibiotics. ESBLs are encoded on plasmids that typically carry other resistance genes which provide activity against aminoglycosides, sulfonamides and quinolones, making the bacteria that acquire these plasmids multidrug resistant.

Conclusion: The isolates of *E .co*li were sensitive to penicillin, ciprofloxacin, and levofloxacin, and they should be taken as first line drugs in the treatment of UTI cases in the environment.

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