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Detection of Antibiotic Resistance Genes in Some Multiple Antibiotic Resistant *E. coli* from Apparently Healthy Pregnant Women

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Authors' contributions

This work was carried out in collaboration among all authors. Author OCA wrote the protocol and the first draft of the manuscript. Author AJFF designed the study, authors RO and GO managed the analyses of the study. All authors read and approved the final manuscript.

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ABSTRACT

The emerging drug resistance, especially among the *Escherichia coli* (*E.coli*) isolates from pregnant women, spread rapidly within the community. Urinary tract infection (UTI) is a well-known bacterial infection posing serious health problem in pregnant women. Also, multi-drug resistance is becoming rampant, and it is of serious public health concern. Treatment of *E. coli* is now a challenge 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 antibiotic resistance genes in some multiple antibiotic resistant *E.coli* from apparently healthy pregnant women in Osun State. A cross-sectional study design was used to collect 150 mid-stream urine samples from apparently healthy pregnant women from March, 2018 to September, 2018. A well structured questionnaire and informed consent were used for data collection. Standard loop technique was used to place 0.001 ml of urine on Cysteine Lactose Electrolyte Deficient (CLED) medium, Blood agar, 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 the resistant genes was done using PCR techniques. The ages of women enrolled in this study ranges from 22 to 42

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years (mean ± standard deviation = 31 ± 4.7 years). *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.

Keywords: Antibiotic resistance; E. coli; pregnant women; treatment.

1. INTRODUCTION

Treatment of UTI is more difficult due to emergence of drug-resistant uropathogens against common antimicrobial agents [1-2]. In Nigeria, drug abuse and misuse are very common among unskilled practitioners, drug consumers and health care providers. Further studies also reveal how drug resistance is seriously affecting the management of already treated infections, which can lead to increased morbidity, mortality and cost of treatment [3-5]. UTI prevalence is increased by several factors such as poor socioeconomic status, which poses a very big risk for UTI infection, as a fivefold increased risk has been identified in indigent patients [6-9]. Some other risk factors may include diabetes mellitus, rise in age, poor perineal hygiene, high parity, anatomic or functional urinary tract abnormality, history of recurrent UTI, and frequent sexual activity among others [9-11].

Treatment of UTI among pregnant women is important for keeping up with the goal of safe motherhood initiative; to ensure women safely go through pregnancy state and childbirth and also produce healthy babies. The problem of antibiotic resistance is a serious issue in treating infectious diseases worldwide [12-14].

The rate of *E. coli* infection among women is alarming, thereby posing a very big health issue among women generally. *Escherichia coli* is the major incriminating pathogen causing UTI. Lack of proper diagnostic tools may result in 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.

The aim of the study was to detect antibiotic resistance genes in some multiple antibiotic resistant *E. coli* isolates from apparently healthy pregnant women.

2. MATERIALS AND METHODS

2.1 Study Area

The study was carried out in Osogbo in Osun State from March 2018 to September 2018. The health facilities selected for sampling were Onward specialist hospital, Agunbelewo, Osogbo and Primary health centre, Atelewo, Osogbo.

2.2 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).

2.3 Study Population

The subjects employed include asymptomatic pregnant women that attended antenatal clinic (ANC) during the study period and those who did not initiate antimicrobial drug therapy for about 2 weeks prior sampling.

2.4 Sampling Methods

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).

2.5 Materials

The media employed for the study include: nutrient agar (HIMEDIA), Muller-Hinton agar (HIMEDIA), MacConkey agar (HIMEDIA). All the reagents used were of analytical grade. The antibiotic discs (Oxoid) employed include: 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).

2.6 Sample Collection

One hundred and fifty midstream urine (MSU) samples were obtained from pregnant women that attended antenatal clinic after proper explanation about collection process. The bottles were appropriately labelled and given identification number before transporting to the Medical Microbiology and Parasitology Laboratory in Osogbo for immediate processing.

2.7 Isolation of Bacteria

Exactly 0.001 ml of urine was used to inoculate Cysteine lactose electrolyte deficient (CLED) medium using standard loop technique. Also, blood agar plates and MacConkey agar plates were inoculated, and all plates incubated for 24 hrs at 37 °C [13-14]. Colony count was done to quantify the isolates. UTI diagnosis is defined based on significant colony count of $=10^5$ CFU/ml as for Gram-negative and in Gram-positive organisms [15]. Colonies were subcultured on fresh agar plates by successive streaking, incubated at 37 °C for 24 hrs to obtain pure culture of isolates [16].

2.8 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), Tetrecycline (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.

2.9 Molecular Detection of Resistant Isolates

2.9.1 Extraction of DNA

The DNA molecules of the *E. coli* isolates were extracted using boiling method.

2.9.2 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

3. RESULTS

3.1 Significant Bacteriuria (10^5) Cases from Pregnant Women Urine Samples

There were 150 pregnant women enrolled for the study. The age of pregnant women considered for this work ranged from 22 to 42 years with a mean age of 32 years (Standard Deviation [SD] = 4.7). From 150 urine samples, 21(14.5 %) (95% CI: 14.4–23.54 %) were culture positive with colony count of over 10^5 CFU/ml.

3.2 Distribution of Isolates from the Pregnant Women

Of the 8 *E.coli* isolates, 3 (37.5 %) were from private hospital and the 5 (62.5 %) remaining were from peri-urban government hospital.

3.3 Antimicrobial Susceptibility Pattern of Bacterial Isolates

Bacterial urinary isolates from UTI patients showed presence of much antimicrobial resistance, both single and multiple against the commonly recommended drugs. *E. coli,* being the predominant UTI causative agent, had high resistance pattern to ampicillin, but low resistance towards ciprofloxacin and penicillin (Table 2). Every one of the isolates of *E. coli* is sensitive to levofloxacin, and most showed resistance to Meropenem (Table 3).

3.4 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), Resistance to more than two classes of antimicrobial drugs, was present among all *E. coli* isolates (100%) from the various sources (Table 4). All the bacterial isolates showed resistance to more than one of the antimicrobials.

3.5 Gel Electrophoresis of PCR Amplified Product of *VIM* Gene

Fig. 1 shows Agarose gel electrophoretogram of (VIM) *Escherichia coli* after PCR analysis. *Escherichia coli* isolates bands at 390 bp, while the size of the marker is 100 bp.

3.6 Gel Electrophoresis of PCR Amplified Product of *bla ctx-M* Gene

Fig. 2 shows the Agarose gel electrophoretogram of *ctx-M*–type β-lactamases *Escherichia coli* after PCR analysis. *Escherichia coli* isolates bands at 585 bp, while the ladder is 100 bp.

3.7 Gel Electrophoresis of PCR Amplified Product of *TEM* Gene

Fig. 3 shows the Agarose gel electrophoretogram of TEM *Escherichia coli* after PCR analysis which bands at 517 bp, with 100bp ladder.

Resistance genes in *VIM* 390 bp, *bla ctx-M* 585 bp and *TEM* 517 bp were detected in some of the representative *E. coli* isolates by gene profile.

4. DISCUSSION

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.

Table 2. Antibiogram of Bacterial Isolates

Antimicrobial agent (Conc.)	Antibiotic Effect on *E. coli* isolates.) n (%)	
	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)	0 (0 %)	
Ciprofloxacin (5 µg)	2 (25 %)	6 (65 %)

KEY: R = Resistant, S= Sensitive

Table 3. Frequency of antibiotic resistance

Antibiotic	N=%
PEN	75
CTX	50
TET	0
MEM	25
PEN	75
CTX	50
TET	0
MEM	25

The result shows that all (100 %) the *E. coli* isolates showed sensitivity to Levofloxacin, 25 % to Ampicilin, 62.5 % to penicillin 37.5 % to cefotaxime, 50 % to Cefuroxime, 65% to Ciprofloxacin, but none was sensitive to Meropenem.

The antibiotic susceptibility pattern in this work showed Levofloxacin as having highest effectiveness for in-vitro testing against isolates of *E. coli*, proceeded by Ciproflaxin, such results as with quinolones have also been given by other authors [17]. This low pathogenic resistance can be due to quinolones being relatively new types of antibiotics which have not yet been abusively used in this part of the world. More so, quinolones are being indicated as effective for treating urinary tract infection of Gram-negative microbial origin like *E. coli, P. aeruginosa, Serratia spp.,* and *Proteus spp.* [18-20]. 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. [21] and Alikhani et al. [22].

Table 4. Pattern of antibiotic resistant

Sample Source	Antibiotics				
Onward Specialist Hosp. (Private Hosp.)					
Isolate 1	PEN	AMC	TET +	MEM	
Isolate 2	AMC	TET	MEM		
Isolate 3	AMC	CTX	TET	MEM	
Primary Health Centre, Atelewo (Govt. Hosp.)					
Isolate 1	PEN	CTX	TET	MEM	CIP
Isolate 2	CPX	TET	MEM		
Isolate 3	AMC	CTX	CPX	MEM	CIP
Isolate 4	AMC	CTX	CPX	MEM	
Isolate 5	PEN	AMC	CTX	CPX	TET MEM

Key: PEN- Penicillin, AMC- Ampicillin, CTX- Cefotaxime, CPX- Cefuroxime, CIP- Ciprofloxacin, TET- Tetrecycline, LEV- Levofloxacin, MEM- MeropenemS- Sensitive, R- Resistant

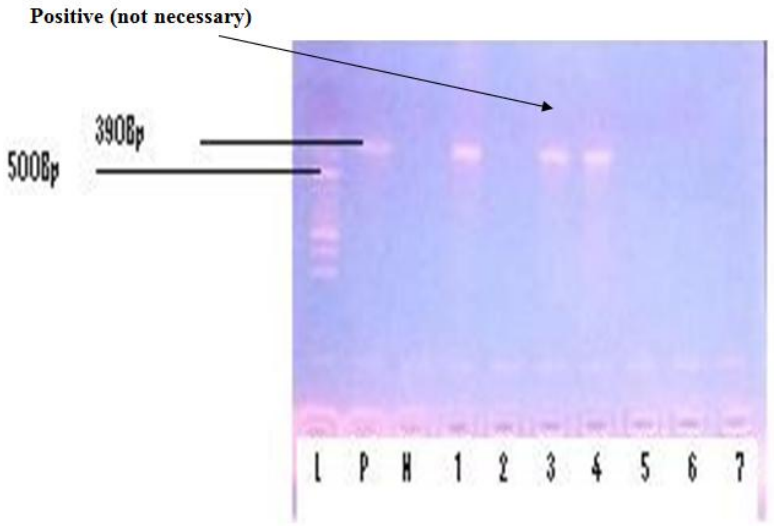

Fig. 1. Agarose gel electrophoretogram of (VIM) *Escherichia coli* isolates from pregnant and non-pregnant women

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

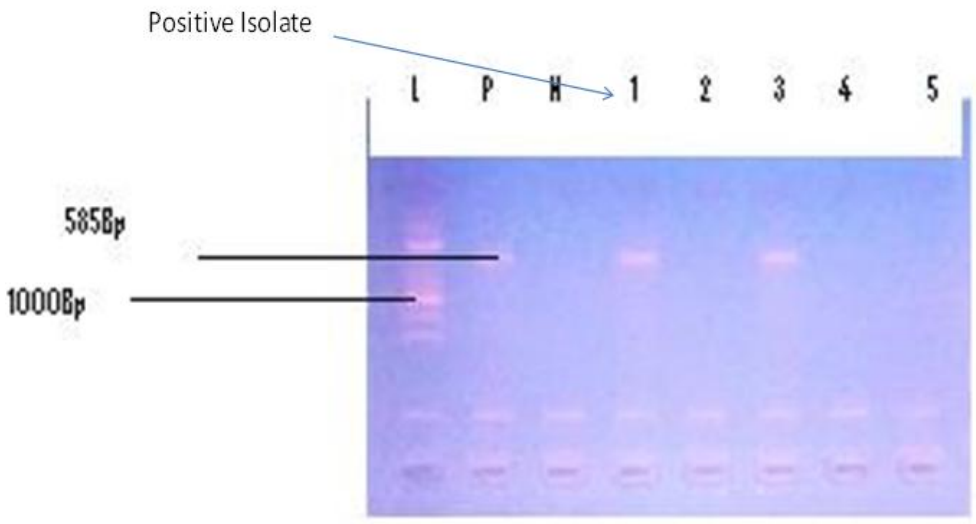

Fig. 2. Agarose gel electrophoretogram of *ctx-M*–type β-lactamases *Escherichia coli* isolates from pregnant and non-pregnant women. *Escherichia coli* isolates which banded at 585 bp

Key: L= 100 bp ladder; P= Positive isolates = 1, 3; N= Negative isolates

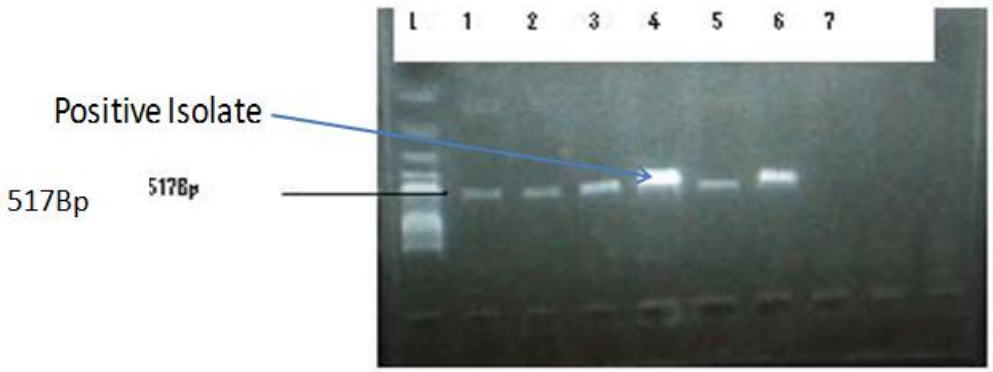

Fig. 3. Agarose gel electrophoretogram of TEM Escherichia coli isolates from pregnant and non-pregnant women

Key: L= Ladder, Positive isolates= 1, 2, 3, 4, 5, 6; Negative isolate= 7

Of all isolates of *E. coli*, 3, 4 and 6 showed positivity for the *VIM, ctx-M* and *TEM* genes respectively. Edelstein and colleagues reported that *ctx-M* beta lactamases have a destructive effect on Cefuroxime. The detection of *VIM* suggested carbapenem-resistance gene being present, and TEM beta-lactamases production.

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. 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 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. [23], [24-26]. The presence of MDR therefore reduces the number of available therapeutic antibiotics that can be used against *E.coli* [27-35].

The isolates exhibited different resistance patterns, some were resistant to three, four or more antibiotics. In the first study center, all the isolates were resistant to ampicillin, tetracycline and meropenem.

However, larger proportion of all the isolates were quite sensitive to penicillin, ciprofloxacin, and levofloxacin, and they should be taken as first line drugs in the treatment. of UTI cases in the environment.

5. CONCLUSION

This study showed an overall highly prevalent of UTI among pregnant women. *E. coli* showed high resistance to the generally prescribed antibiotics. Low resistance levels were discovered for cefuroxime, ciprofloxacin and levofloxacin, thus can serve as UTI empirical therapy in the area of study.

Multi-drug resistant bacteria of the urinary tract have widespread distribution among the pregnant women from the area of study. Multi-drug resistance also was observed, all isolates of *E. coli* were resistant towards Cefotaxime, ampicillin and meropenem. Some isolates of *E. coli* were positive for the *VIM, ctx-M* and *TEM* resistant genes. C

6. RECOMMENDATION

Health education, collaborative and continuous UTI surveillance and pattern of antimicrobial resistance are essential in the reduction of the impact of both symptomatic and asymptomatic bacteriuria, and multiple drug resistant pathogens among pregnant women.

CONSENT AND ETHICAL APPROVAL

This work was performed according to University ethics committee code of conduct and informed consents were gotten from the subjects.

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