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Phenotypic and molecular characterization of multiple-resistant gram-negative bacteria in urine of pregnant women attending antenatal clinic of Mother and Child hospital, Ondo, Nigeria

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Phenotypic and molecular characterization of multiple antibiotic resistant Gram-negative bacteria in urine samples of pregnant women in Mother and Child Hospital, Nigeria was reported. In the study, 407 apparently healthy pregnant women were recruited. Structured questionnaire was administered to the patients to obtain their socio-demographic information and the medical history. Urine samples were collected, processed and analysed using standard microbiological procedures. Detailed identification of the bacteria isolates was done using biochemical characterization using Bergey's Manual of Determinative Bacteriology and Analytical Profile Index (API) Kit. The antimicrobial susceptibility testing of the bacteria isolates was carried out using the Kirby-Bauer's disk diffusion technique. Detection of the beta lactamase resistance genes (bla $_{\text{CTX-M}}$ and *Tet* A) was done by polymerase chain reactions (PCR) with appropriate primers. The following Gram-negative bacteria were recovered comprising *Pseudomonas aeruginosa* 48 (34.0%), *Escherichia coli* 30 (21.3%), *Klebsiella* sp. 27 (19.1%), *Proteus* sp. 15 (10.6%), *Salmonella* sp. 8 (5.7%), *Providencia rettgeri,* 4 (2.8%) and *Enterobacter cloacae* 4(2.8%) and other enterobacteriaceae 5 (3.5%). Resistance of the isolates to antibiotics used varied greatly among the isolates. Resistance to antibiotics was highest with *P. aeruginosa* having 100% to augumentin, tetracylines, amoxicillin, nitrofurantoin, cotrimoxazole, ceftriazone, cefixime (97.9%) and cefuroxime (95.8%). There was diversity in the multiple antibiotic resistance (MAR) patterns among the isolates with 12 different MAR patterns observed. The selected *P. aeruginosa* profiled for resistance genes harboured *bla-CTX-M* (585bp) and *Tet* A (954bp) genes. The multiple antibiotic resistant bacteria recovered could pose great health challenge to the pregnant women and the unborn foetus.

Key words: Gram negative bacteria, antibiotics, enterobacteriaceae, resistance genes.

INTRODUCTION

Bacteriuria can be defined as the appearance or presence of bacteria causing diseases in the urethra, bladder, and pelvis of the kidney. Bacteria such as *Escherichia coli*, *Enterococcus faecalis Klebsiella* species, *Proteus mirabilis*, *Pseudomonas aeruginosa* and *Streptococcus agalactiae* are some of the causative agent of urinary tract infections (UTI). Blockage of the urinary tract, catheter usage, deficiency in oestrogen, genetic predisposition and sexual intercourse are predominant risk factors for urinary tract infection (Tigabu et al., 2020). Asymptomatic bacteriuria (ASB) is the detection of 10^5CFU/ml of one or more 46 bacterial species, irrespective of pyuria, in a urine specimen from a pregnant women patient without any symptoms of a urinary tract infection (UTI) (Willey et al., 2020). The relatively upsurge of ASB in pregnancy, the consequences encountered by the women and their pregnancies, avoidance of treatment with undesired outcomes, screening and treatment of ASB in pregnancy with justification. There are variations in the frequency of pathogens isolated and antimicrobial resistance patterns based on different geographical regions. Asymptomatic bacteriuria occurs in 2 to 15% of pregnant women. Therefore if the ASB remains untreated, over 30% of mothers will develop acute pyelonephritis, and this has been associated with low birth weight and premature birth (Sujatha and Nawani, 2014; Smaill and Vazquez, 2019).

It is recommended that screening for bacteriuria should routinely be undertaken in the first trimester of pregnancy to identify women who are at risk in order to prevent undesirable end result as bacteriuria which is one of the risk factor in pregnant women. The importance of the microbiological analysis of urine samples collected from patients that appears healthy cannot be over emphasized.

Multiple antibiotic resistant Gram-negative bacteria are bacteria which have developed resistant to many common commercial used antibiotics. Their habitat is in the bowel and therefore causes no harm or any problems but can cause infections in the urine, skin wounds and blood when left untreated. These multidrug resistant Gram-negative bacteria infections pose a serious threat in the clinical settings with limits to the choice of antibiotics in the treatment and management of infections in patients that are hospitalized, and more especially patients in intensive care unit. Overuse and misuse of antibiotics and problems and wrong infection control practices have led to the development of multiple resistant Gram-negative bacteria infections. This study investigates into the phenotypic and molecular characterization of multiple resistant Gram-negative bacteria among pregnant women attending antenatal of Mother and Child Hospital in Ondo, Nigeria.

METHODOLOGY

Isolation of bacteria

Urine samples of four hundred and seven apparently healthy pregnant women attending antenatal clinic at Mother and Child hospital in Ondo Town were collected between July 2015 and January 2016. Samples were cultured on Centrimide and MacConkey agar (Lab M Ltd, UK) by streaked plate method, incubated at 37°Cfor 24 h for isolation of *P. aeruginosa* and other Gram-negative bacteria. Preliminary identification of isolates was by morphological, cultural characteristics and biochemical tests. Bacteria isolates were further re-confirmed using analytical profile index (API) 20E test kit (bioMérieux, Inc., France).

Antibiotic susceptibility testing

Susceptibility test of the isolates to antibiotics was carried out by the Kirby-Bauer's disc diffusion method (Bauer et al., 1966). Antibiotic discs (Abtek Biological limited, UK) which include; gentamycin (10 µg), augmentin (30 µg), ceftazidime (30 µg), ceftriaxone (30 µg), cefuroxime (30 µg), nitrofurantoin (200 µg), cotrimoxazole (25 µg), ofloxacin (5 µg), amoxicilin (25 µg), tetracycline (30 gµ), ciprofloxacin (5 µg), ofloxacin (5 µg), ceftriazone (30 µg) and cefixime (5 µg) were carefully placed on Mueller-Hinton agar plates previously seeded with 24 h old culture (0.5 Mcfarland's standard- 10^7cfu/ml). The plates were incubated at 37°C for 24 h and diameter of zone of inhibition was measured by a transparent calibrated ruler to the nearest millimetre and the results interpreted according to the guidelines of Clinical Laboratory Standard (CLSI, 2013). Multiple antibiotic resistance was defined as resistance to more than two class of antibiotics.

Molecular characterization of multiple antibiotic resistant isolates

Twelve representative multiple antibiotic resistant bacterial isolates selected on the basis of their antibiotype were profiled for detection of resistance *bla* CTX (585bp) and *Tet* A (954bp) genes by polymerase chain reaction (PCR) using appropriate primers as depicted in Table 1. The DNA of the bacteria isolates was extracted using boiling method at 100°C for 7 min in water bath, cold shocked in ice for 2 min. The PCR thermocycling conditions include initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s for *bla* CTX (585 bp) gene and 43°C for 30 s for *Tet* A (954 bp) gene, extension at 72°C for 1 min and final extension at same temperature for 5 min. The bands were then visualized with a short wave ultraviolet trans illuminator and photographed gel bioimaging system.

RESULTS

Table 2 shows the baseline characteristics of the subjects. The women recruited were between the age brackets of 15 and 49 years. Among the 407 pregnant women, 36(8.8%) was in their first trimester, 171(42.0%) in second trimester and 200(49.1%) in third trimester. Six (1.5%) of the subjects was single, while 401(98.5%) was married. Also, 37(9.1%) was students, 75(18.4%) was public or civil servants, 6(1.5%) was professionals,

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Table 1. Primers used for the detection of the resistance genes in the selected isolates.

Primer	Sequence 5^1 -3^1	Gene	Product size (bp)	References
CTX-M F CTX-M R	CGATGTGCAGTACCAGTAA TTAGTGACCAGAATAAGCGG	bla CTX-M	585	Colom et al. (2003)
TET-A	F:GCGCTNTATGCGTTGATGCA R: ATGTGTCCTGGATTCCT	TET-A	246	Adesiyan et al. (2019)

Table 2. Baseline characteristics of the pregnant women.

Characteristics	Cases n (%)
Marital status:	
Single	6 (1.5)
Married	401 (98.5)
Patients status	
Religion	
Christianity	371 (91.2).
Islamic	36 (8.8).
Occupation	
Civil/public servants	75 (18.4)
Professional/managers	6 (1.5)
Farming/artisans/trading	253 (62.2)
Students	37 (9.1)
Dependant/unemployed	16 (3.9)
Others	20 (4.9)
Pregnancy trimesters	
First trimester	36 (8.8)
Second trimester	171 (42.0)
Third trimester	200 (49.1)
Age group	
15-19	15 (3.7)
20-29	221 (54.3)
30-39	156 (38.3)
40-49	15 (3.7)

253(54.4%) was artisans or traders and 6(3.9%) was dependent or unemployed.

Five pregnant women within the age bracket 21-32 years suffering from urinary tract infection served as control and were all in their second and third trimesters. Table 3 depicted the percentage distribution of the bacteria isolates recovered viz; *P. aeruginosa* (34.0%), *E. coli* (21.3%), *Proteus* sp (10.6%), *Salmonella* sp (5.7%), *Klebsiella* sp (19.1%), *P. rettgeri* (2.8%), *E. cloacae* (2.8%) and other enterobacteriaceae 5 (3.5%). Table 4 shows the percentage occurrence of bacteria isolated from pregnant women in relation to their occupation. The percentage distribution of the bacterial isolates varies with occupation. There was no bacterial isolates recovered from pregnant women who are professionals/ managers.

Table 5 shows the antibiotic resistance profiles of the bacterial isolates. Resistance to antibiotics varies greatly among the organisms. Resistance to beta-lactam class of antibiotics was generally high among the organisms. P. aeruginosa showed highest resistance most antibiotics tested namely augmentin (100%), tetracyclines (100%),

Table 3. Percentage Distribution of Bacterial Isolates in the Urine Samples of Pregnant Women at Mother and Child Hospital Ondo.

Names of organisms	Total number of isolates	No of occurrence	Percentage
P. aeruginosa	141	48	(34.0
E. coli	141	30	(21.3)
Salmonella sp.	141	8	(5.7)
Proteus sp.	141	15	(10.6)
K. pneumoniae	141	27	(19.1)
E. cloacae	141	4	(2.8)
P. rettgeri	141	4	(2.8)
Other enterobacteriaceae	141	5	(3.5)

Table 4. Percentage distribution of bacterial isolates in relation to occupation of the pregnant women.

Occupation	*Proteus* sp.	*E. coli*	*Klebsiella* sp.	*P. aeruginosa*	*Salmonella* sp.	*P. rettgeri*	*E. cloacae*	Others
Trader	66.7	86.7	74.1	79.2	100	100	100	100
Civil /public servant	33.3	10	-	2.1	-	-	-	-
Dependant	-	3.3	-	2.1	-	-	-	-
Student	-	-	14.8	6.3	-	-	-	-
Professional/managers	-	-	-	-	-	-	-	

amoxicillin (100%), ceftriaxone (100%), cotrimoxazole (100%), nitrofurantoin (95.8%), cefuroxime (95.8%) and cefixime (97.9%). *E.coli* were only resistant to augmentin (93.3%).

Table 6 showed the multiple antibiotic resistance pattern of *P. aeruginosa* and *Klebsiella ornithinolytica* isolates. All *P. aeruginosa* and *K. ornithinolytica* isolates recovered from this study were multidrug resistance. The isolated bacteria were resistant to multiple antibiotics which ranges from four to seven classes. *K. ornithinolytica* (9.5%) were resistant to four class of antibiotics. *P. aeruginosa* (97.9%) were multi-resistant to six different class, 2.1 to 7% and 4.2 to 5% different class of antibiotics. Meanwhile, both *K. ornithinolytica* and *P. aeruginosa* exhibited 2 different multiple antibiotic resistance patterns each (Table 6).

Figures 1 and 2 showed agarose gel electrophoresis of the amplification product coding *bla* CTX (585 bp) and *Tet* A (954) genes in selected MAR *P. aeruginosa* and *K. ornithinolytica* isolates. In Figure 1, seven of the 12 representative isolates that were resistance to beta lactam antibiotics as depicted by Lanes 3, 4, 5, 7, 8, 9, and 12 harboured *blaCTX* resistance gene of molecular weight of 585 bp. Lanes 1-2 represent *K. ornithinolytica* which harboured no *blaCTX* resistance gene. Lane 11 represents the control (patient suffering from urinary tract infection) which also harboured no *blaCTX* resistance gene. Figure 2 shows the agarose gel electrophoresis of *Tet*A genes in selected 12 representatives multiple antibiotic resistant *P. aeruginosa*. Five of the 12 representative isolates that were resistant to tetracycline antibiotics as depicted by Lanes 3, 4, 5, 7 and 8, harboured *Tet* A resistance gene of molecular weight of 945 bp. Lane 11 represents control (patient suffering from urinary tract infection).

DISCUSSION

This study showed the prevalence of 36.4% bacteria amongst the sampled population of 407 pregnant women attending ante-natal clinic at Mother and Child Hospital Ondo town Nigeria. This level of prevalence is at variance to other researchers' reports. Olamijulo et al. (2016) reported a prevalence of 14.6% in a study carried out in 556 pregnant women in Lagos University Teaching Hospital, Nigeria. It is also at variance with Nguefack et al. (2019) who also reported a prevalence of 9.9% in a

Table 5. Resistance profile of the isolates cultured from urine sample of pregnant women.

Organism	Aug (30 µg)	Cpr (5 µg)	Nit (200 µg)	Gen (10 µg)	Ctx (30 µg)	Ofl (5 µg)	Cfx (30 µg)	Cxm (5 µg)	Amx (25 µg)	Tet (5 µg)	Cfr (30 µg)	Cot (25 µg)	S.D
E. coli (n=30)	28(93.3)	0	0	0	0	0	0	0	ND	ND	ND	ND	$p \geq 0.05$
Salmonella sp (n=8)	4(50.0)	0	0	0	8(100)	0	4(50.0)	8(100)	ND	ND	ND	ND	$p \leq 0.05$
E. clocae (n=4)	2(50.0)	0	0	0	0	0	0	0	ND	ND	ND	ND	$p \geq 0.05$
P. retgeri (n=4)	2(50.0)	0	0	0	2(50.0)	0	0	0	ND	ND	ND	ND	$P \leq 0.05$
Kluyvera sp (n=3)	2(66.7)	2(66.7)	0	0	0	0	0	0	ND	ND	ND	ND	$p \geq 0.05$
Klebsiella sp (n=27)	26(96.3)	1(3.7)	1(3.7)	1(3.7)	0	1(3.7)	26(96.3)	27(100)	ND	ND	ND	ND	$p \geq 0.05$
P. aeruginosa (n=48)	48 (100)	0	46 (95.8)	1 (2.1)	0	0	46 (95.8)	45 (93.8)	48 (100)	48 (100)	48 (100)	48 (100)	

Aug= Augmentin, Cpr= Ciprofloxacin, Nit= Nitrofurantoin, Gen= Gentamycin, Ctx= Ceftaxidime, Ofl= Ofloxacin, Cfx= Cefuroxime, Cxm= Cefixime, Amx= Amoxicillin, Tet= Tetracycline, Cfr= Ceftriaxone, Cotrimozaxole, ND= Not Determined, S.D= Standard Deviation.

Table 6. Multiple antibiotic resistance pattern of *P. aeruginosa* and *K. ornithinolytica*.

Name of isolates	No. of classes of antibiotics tested	Multiple resistant pattern	Frequency (n%)	Occurrence of MAR isolates (n%)
K. ornithinolytica	4	AMX, AUG,CRX,OFL	1 (50)	
K. ornithinolytica	4	AMX, AUG,CRX,OFL	1 (50)	2 (100)
P. aeruginosa	5	AUG, AMX, COT, CRX, TET,	47 (97.9)	
P. aeruginosa	7	AUG, AMX, COT, CRX, GEN, NIT, TET	1 (2.1)	48 (100)

COT –Cotrimoxazole 25 µg, CPX –Ciprofloxacin 10 µg, AMX –Amoxycilin 25 µg, OFL –Ofloxacin 5 µg, CRO –Ceftriazone 30 µg, GEN –Gentamycin 10 µg, CRO- ceftriazone 30 µg, AUG –Augmentin 30 µg, NIT- Nitrofurantoin 200 µg, TET –Tetracycline 5 µg, CRX–Cefuroxime 30 µg, CXM- Cefixime 30 µg, CAZ- ceftazidime 30 µg.

three hospitals in the developing country.

The prevalence of bacteria in increasing order in this study, *P. aeruginosa* (34.0), *E. coli* (21.3), *Klebsiella* sp (19.1), *Proteus* sp (10.6), *Salmonella* sp (5.7), other enterobacter (3.5), *Providencia rettgeri* (2.8) and *Enterobacter clocae* (2.8) is in contrast to Olamijulo et al. (2016) who reported that *Klebsiella* sp is the most common pathogen isolated in urine of pregnant women. Gram-negative bacteria are responsible for more than 85% cases of UTI and are the dominant causative agents, also they are the normal flora of the intestinal tract especially the rectum which is very close to the urethral orifice (Anyamene et al., 2002; Obiogbolu, 2004).

Bacteria were predominantly recovered among women of ages (19-32) in their second and third trimesters with none in the first trimester. This finding is quite similar to Durowaiye et al. (2011) who reported the Women in the third trimester were observed to have the highest prevalence 18.2% than those in their second and first trimester. This could be because of changes that occur in the anatomic site and physiology that is been experienced by pregnant women during the stages of pregnancy. Because of the uterus expansion there is increase in the hormonal effects which can together lead to invasion of microorganisms. These hormonal changes reduce the muscular tone of the uterus and induce the mechanical pressure from the gravid uterus and this may leads to urinary stasis which encourages the multiplication of bacteria in urine since urine is an excellent culture media for bacteria growth (Obiogbolu, 2004). Pregnant women within the age of 20-39 years old age group had the highest

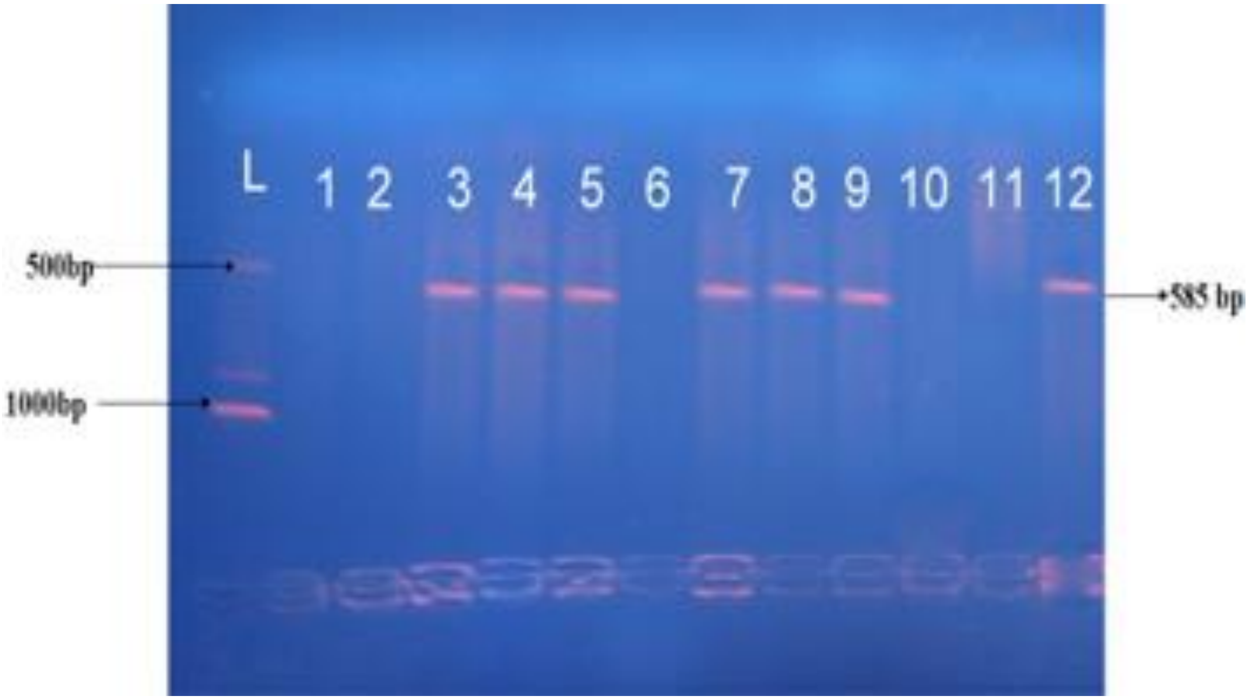

Figure 1. Agarose gel electrophoresis of the amplification product coding blaCTX (585bp) gene in selected MAR *Pseudomonas aeruginosa* isolates and *K. ornithinolytica* isolates. Ladder L: DNA marker 100 bp; Lane 1-2 *K. ornithinolytica*; Lane 3-12: *P. aeruginosa*. L1- K238;L2-K116; L3-P188; L4-P269;L5-P351;L6-P332;L7-091;L8-P355;L9-P372;L10-P081;L11-P311(control);L12-P074 L- Lader, K- *Klebsiella ornithinolytica*, P-*Pseudomonas aeruginosa*.

prevalence of bacteria. This shows that these women are at the risk of developing urinary tract infection in future if not or properly treated. The bacteria are responsible for asymptomatic bacterial infection are of faecal origin and they colonize the periurethral region.

The percentage of *Proteus* sp, *P. retgeri*, *E. coli*, *Klebsiella* sp, *P. aeruginosa*, *Salmonella* sp and *E. clocae* was predominantly high among pregnant women who were farmers/ artisans/traders. This high prevalence among these categories of women could be as a result of consumption of contaminated food, socio-economic status, attitude to personal hygiene and educational exposure.

All the bacterial isolates in this study namely; *K. ornithinolytica*, *E. coli*, *E. clocae*, *Salmonella* sp, and *P. rettgeri* were highly sensitive to nitrofurantoin, gentamycin, and ofloxacin except *P. aeruginosa*. Similarly, *E. coli* were relatively sensitive to ceftaxidime, cefixime, ciprofloxacin, gentamycin, nitrofuraintoin and cefuroxime. This finding is comparable to Olamijulo et al. (2016) who reported in their study that Gram-negative bacteria showed high sensitivity rate to gentamycin and ofloxacin. This could be because gentamycin belongs to the class aminoglycoside where antibody binds to the subunit of the bacteria ribosome, interruption of protein synthesis thereby preventing bacteria from performing vital roles needed for survival. Therefore, gentamycin can be used in the treatment of these Gram-negative bacteria in urinary tract infection.

Antimicrobial susceptibility testing in this study revealed the high resistance of *P. aeruginosa* to amoxicillin which belongs to penicillin class (beta lactam group) by the isolates recovered. Similar results were reported in ast studies by Sabharwal (2012). This could be a result of misuse and excessive usage of antibiotic and increase in the spread of beta lactamase producing isolates. Sensitivity of *P. aeruginosa* to flouroquinolones (ciprofloxacin (100%) and ofloxacin) were significant in this study thereby revealing the potency of the antibiotics against the urinary tract pathogens. However, widespread usage may lead to resistance against fluoroquinolones (Gupta et al., 2005).

P. aeruginosa in this study showed high resistance to nitrofurantoin (95.8%) which is in contrast to a report that nitrofurantoin, is a urinary antiseptic, and found to have a better susceptibility and considered safe by Peterson and Andriole (1997) and Christensen (2000).

The result from the antimicrobial susceptibility profile in this study is closely related to that obtained by Akingbade et al. (2012), who reported high resistance rate of *P. aeruginosa* to the following antibiotics: amoxicillin (92.7%), oxacillin (88.2%), cotrimoxazole (77.3%), erythromycin (72.7%), and tetracyclines (70.9%), while they also reported a relatively low resistance to gentamicin as recorded in the present study. Similarly, they reported a low resistance to ceftazidime (20%),

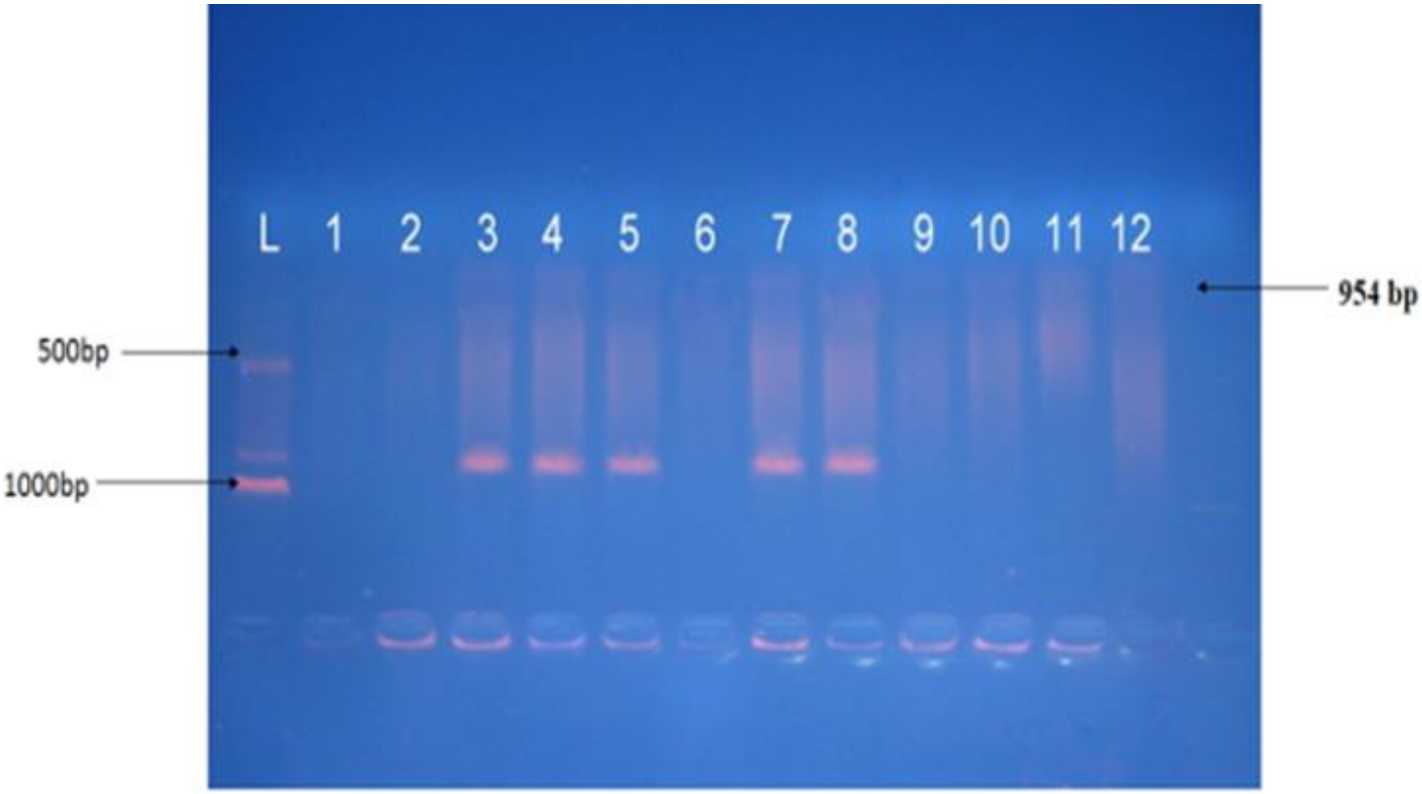

Figure 2. Agarose gel electrophoresis of the amplification product coding *Tet A* (954 bp) gene in selected MAR *P. aeruginosa* isolates. Lanes 1-12: *P. aeruginosa*. Ladder marker 100bp. Ladder, 1= 188, 2=332, 3=372, 4=269, 5=351, 6=311(control), 7=P091, 8=355, 9=081, 19=074, 11=011 and lastly 12=181. L- Lader, P-*Pseudomonas aeruginosa*.

gentamicin (26.4%) by *P. aeruginosa*, while a sharp contrast pattern was observed in the present study with high sensitivity to ceftazidime (97.9%), gentamycin (97.9%) and ciprofloxacin (100%), but high resistance to tetracycline (100%), amoxicillin (100%), cotrimoxazole (100%) and ceftriaxone (100%). Also, Akingbade et al. (2012) recorded a high resistance to ofloxacin (60.0%) which is in contrast to the present study in which a relatively high sensitivity to ofloxacin (100%) was observed.

P. aeruginosa is known to be an opportunistic pathogen which is a leading cause of morbidity and mortality rate in immunocompromised individuals including pregnant women. *P. aeruginosa* has become extremely difficult to eradication because of its remarkable capacity to resist different antibiotics. Strains of *P. aeruginosa* have been known to utilize high levels of intrinsic and acquired resistance mechanisms to attack most antibiotics. Biofilm-mediated resistance and formation of multidrug-tolerant persister cells have been a recent characterized mechanism for adaptive antibiotic resistance which is responsible for recalcitrance and relapse of infections (Pang et al., 2019). The discovery and development of alternative therapeutic strategies that present novel avenues to fight *P. aeruginosa* infections are increasingly demanded and gaining more attention. According to findings from this study, all *P. aeruginosa* recovered from the patients were multiple antibiotic resistant. This high prevalence of multiple antibiotic resistance (MAR) is more alarming and its consistent with previous researchers' findings (Pharmd et al., 2018; Shah et al., 2015). Resistivity of the isolates to various antibiotics could be because of overuse of antibiotics and self-medication resulting in drug resistance especially by *P. aeruginosa*.

The detection of beta-lactamase resistance genes in *P. aeruginosa* in the study is undoubtedly partly responsible for the high resistance rate phenotypically observed particularly against most of the beta-lactams antibiotics used. These genes code for acquired extended spectrum beta-lactamases which are involved in the resistance against beta-lactams and are located in transferable genetic elements such as plasmids or transposons of the organisms (Giedraitienė et al., 2011) and often on integrons (Kotsakis et al., 2010; Zhao et al., 2009; Nordmann et al., 2012). Ogbolu et al. (2013) reported a prevalence of 30.8, 15.4, and 23.1% for bla_{TEM}, bla_{SHV} and $bla_{\text{CTX-M}}$ genes, respectively from *P. aeruginosa* isolates recovered in their studies. In this study, $bla_{\text{CTX-M}}$ was also detected in the organism. This study detected the presence of *Tet*A resistance gene among *P.*

aeruginosa.

In conclusion, the varieties of bacteria isolated in this study have great implication on the health status of pregnant women and their unborn fetuses. Antibiotics such as gentamycin, ciprofloxacin, ofloxacin and ceftazidime play a great role in the treatment of bacteria in pregnant women. Health education on personal hygiene should be emphasized by the physicians of antenatal care to all pregnant women, especially those of low socio-economic level.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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