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PHENOTYPIC AND MOLECULAR DETECTION OF EXTENDED SPECTRUM BETA-LACTAMASES PRODUCING-BACTERIA ISOLATED FROM PREGNANT WOMEN WITH GENITAL TRACT INFECTION IN DUHOK GOVERNORATE

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ABSTRACT:
Beta-lactamase producing bacteria have a worldwide distribution with a high degree of prevalence in both community and hospital. Furthermore, multidrug resistant (MDR) and extended spectrum β-lactamases (ESBL) producing bacterial isolates from women patients may limit treatment options available. This study was designed to determine the frequency of bacterial isolates associated with genital tract infection in pregnant women and their antimicrobial resistance profile and to assess the prevalence of extended spectrum β-lactamases producing bacteria. Demonstrating the β-lactamase genes (bla_{TEM}, bla_{SHV} and bla_{CTX-M}) by using polymerase chain reaction (PCR) assay with specific primers, was carried out on patients who were admitted to Maternity and Obstetric Hospital in Duhok city from November 2018 to October 2019. A total of 100 high vaginal swabs were collected from pregnant women patients between the ages 18-45 years. All clinical samples were cultured and standard microbiological methods were used to identify bacterial isolates, then confirmed by Vitek®2 compact automated system. All gram negative bacterial isolates were studied phenotypically and genotypically for extended spectrum β-lactamases-production. Out of 100 vaginal swabs, 88% confirmed positive culture; 90.9% of which were bacterial isolates. From the total bacterial isolates, 38.8% were gram negative bacteria, with a predominant 54.8% *Klebsiella pneumoniae* followed by *Escherichia coli* 35.5%. 54.8% of the isolates were characterized as multidrug resistance isolates, 29% isolates were extensively drug resistance, and no pan drug resistance were detected. Among these, the commonest extended spectrum β-lactamases producing isolates were *Escherichia coli* 81.8% followed by, *Klebsiella pneumoniae* 58.8%. Extended spectrum β-lactamases-producing isolates have showed significantly higher resistance than non- extended spectrum β-lactamases producing isolates to third and fourth generation cephalosporins. CTX-M was the most common β-lactamase gene 73.7% among extended spectrum β-lactamase producing strains, followed by bla_{SHV}, 57.9% and bla_{TEM} 52.6%, 21.1% had combination of all *bla* genes, 15.8% had CTX-M only and combination of bla_{CTX-M} with bla_{SHV} and bla_{TEM}. 10.5% among extended spectrum β-lactamases producing isolates carried SHV type only and in combination with TEM type while TEM gene were observed in 5.3%. We concluded that the drug resistant isolates were common, worryingly high and it may limit treatment options available. In this study a high level of the bla_{CTX-M} gene was demonstrated among extended spectrum β-lactamases producing isolates.

KEYWORDS: Vaginal Infection, Pregnant Women, MDR, XDR, ESBL, Iraq.

1. INTRODUCTION

Vaginitis is a term used to describe the infectious disease and other inflammatory conditions affecting the vaginal mucosa which are characterized by vaginal discharge, burning, itching, and discomfort; which usually cause complaints among patients who attend obstetrics and gynecology clinics (Donders *et al*., 2011). It occurs when the normal vaginal *Lactobacillus spp.* dominated with aerobic pathogens such as *E.faecalis, E.coli, and S.aureus that trigger a localized vaginal inflammatory immune response (*Sangeetha *et al*., 2015). Vaginitis is a health problem in pregnant women that result in complications and serious medical consequences, such as Premature rupture of membranes (PROM), Preterm labor, Postpartum endometritis, Intra-amniotic infection, Spontaneous abortion and low birth weight (Kaambo *et al*., 2018). Consequently, diagnosing and treating vaginitis during the pregnancy may help decrease a risk of adverse pregnancy outcomes (Tang *et al.,* 2020). The emergency of the resistance to antimicrobial agents constantly develops seriously, affecting the assessment and infectious treatment in the community, and the health-related setting. ESBLs are enzymes which are capable of hydrolyzing broad spectrum cephalosporins, penicillins, and monobactams (Fernando *et al*., 2017). The production of ESBLs is commonly found among gram-negative bacteria including *E. coli, Kl. pneumoniae, P. mirabilis,* and *Ps. aeruginosa* (Ogefere *et al.,* 2015). Additionally, ESBL producing bacteria exhibit co-resistance to several other antibiotic groups which may limit treatment options available. Little information is available in the Kurdistan region of Iraq about the colonization of ESBL-producing bacteria in pregnant women. Therefore, this study was carried out in Duhok-Iraq to screen pregnant-women for ESBLs-producing bacteria and determine their phenotypic, and genotypic characterization, and to study the prevalence of various ESBL genotype patterns between ESBLs-producing bacteria.

2. PATIENTS AND METHODS

2.1. Patients and study design

One hundred High Vaginal Swabs (HVS) were gathered from pregnant women between the age (18-45) years; clinically having abnormal vaginal discharge, burning, itching, and lower

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abdomen pain, who attended to Maternity and Obstetric hospital in Duhok city between November 2018 and October 2019. All high vaginal swabs conducted under medical staff supervision were done using a sterile speculum (Hi Tech Zone, China) and swab stick. All pregnant patients who had not received any antibiotics were involved in the present study.

2.2. Sample collection

Two vaginal swabs were taken from each pregnant patient by a gynecologist by gently pressing the swab into the vaginal sidewall and rotating the swab several times to thoroughly coat the swab. The first swab was immediately and carefully transferred into amies transport media (cultiplast tampone swab, Italy) for culture investigation, while the second swab was directly transferred by adding sterile normal saline for measuring the pH, direct microscopic examination, and gram staining; then labeled with the name of the patient's, identification number, age, date, and time of collection as well as full information that were taken directly from the patients.

2.3. Culture and identification

All vaginal swabs were cultured on the following culture media: Blood, MacConkey, Chocolate agar, and selective media (Lab M, UK) for bacterial isolation. The plates were incubated aerobically and anaerobically at 37 ºC for 24 hours. While Sabouraud dextrose agar (HIMEDIA, India) is selective for fungi, then plates were incubated under aerobic conditions for 24-48 hours at 35ºC. Standard microbiological methods were used to identify bacterial isolates: Colony morphology, Gram stain, and Biochemical test (Talaiekhozani *et al.*, 2015). Species identification for all obtained bacterial isolates were carried out by Vitek®2 compact automated system (BioMerieux®, France) using GN-ID (REF 21341) cards for identifying gram negative bacteria according to (Pincus, 2010).

2.4. Antimicrobial susceptibility testing

All bacterial isolates were tested against seventeen antibiotic disks (Bioanalyse, Turkey). Antimicrobial susceptibility was determined by the Kirby-Bauer disk diffusion method on Mueller–Hinton agar (HIMEDIA, India). The zone of inhibition was measured according to Clinical and Laboratory Standard Institute guidelines (CLSI, 2018) for the following antimicrobial disks: Amikacin, Gentamicin, Netilmicin, Imipenem, Ertapenem, Meropenem, Ceftriaxone, Cefuroxime, Ceftazidime, Cefepime, Ampicillin, Aztreonam, Piperacillin, Piperacillin–tazobactam, Amoxicillin clavulanic acid, Trimethoprim, and Ciprofloxacin. Multidrug resistant, extensively drug resistant and pan drug resistant strains were detected according to European Centre for Disease Prevention and Control (ECDC) and the Centre for Disease Control and Prevention (CDC); MDR refer to the isolates that resistance to at least one agent in three or more antimicrobial classes. Any isolate that remains sensitive to only one or two class of antibiotics it is characterized XDR. While PDR is defined as non-susceptibility to all agents in all antimicrobial classes (Magiorakos *et al.*, 2012).

2.5. Phenotypic detection of Extend Spectrum β-Lactamases

2.5.1. Double-disk synergy test: The confirmation of ESBL-producing gram-negative isolates was done by using the Double-Disk Synergy Test on cultured (Muller Hinton agar) plate. Third generation Cephalosporin (Cefotaxime 30 µg, Ceftriaxone 30 µg, and Ceftazidime 30 µg) were put 20 mm (center to center) away from the Amoxicillin clavulanic acid disk (20 µg Amoxicillin and 10 µg clavulanic acid) on the same plate, then incubated overnight at 37ºC. Extension of inhibition zone of any type of 3rd generation Cephalosporin toward the disk of Amoxicillin clavulanic acid was considered as positive to the ESBL production (Biswas *et al.*, 2013).

2.5.2. ESBL CHROMagar: All gram negative bacteria were cultured on CHROM agar ESBL (Conda pronadisa, Spain) to detect ESBL producer isolates after adding ESBL supplement (CAT: 6042); then inoculated at 37ºC for 24 hours. *E.coli* and *Kl.oxytoca* produced pink to burgundy colonies, while *Kl.pneumoniae; Enterobacter* and *Serratia* blue to blue-green coloration colonies; *Proteus* produce dark to light brown coloration colonies; the colorless colonies considered as ESBL producing *Pseudomonas* and *Acinetobacter* (Gazin *et al.*, 2012)

2.6. Genotypic detection of Extend Spectrum β-Lactamases

2.6.1. Bacterial genomic DNA extraction: Genomic DNA from all ESBL producing isolates was extracted using a commercial DNA Purification Kit (Promega, USA) as recommended by the manufacturer. Briefly, 1 ml of an overnight culture was centrifugated at 13000 xg for 2 minutes, to pellet the cells. After that, (600) µl of nuclei lysis solution was added and gently pipet until the cell pellet was suspended and incubated for 5 minutes at 80 ºC, then cool at room temperature. Three µl of RNase solution is added to the cell lysate and incubated for 40 minutes at 37 ºC and cool the sample to room temperature. Then, 200 µl of protein precipitation solution was added and mixed vigorously for 20 seconds. The sample was cooled on ice for 5 minutes and centrifugated for 3 minutes. Transfer the supernatant to tube containing 600 µl isopropanol, and gently mixed by inversion until forming thread-like strands of DNA, then centrifuged for 2 minutes. It is then pour off the supernatant and then added 600 µl 70% ethanol and gently invert the tube several times and centrifuged for 2 minutes, then aspirate the ethanol and allow the pellet to air dry for 10 minutes. Finally, 100 µl of DNA rehydration solution is added and incubated overnight at 4ºC. Rehydrated DNA was stored at -20 ºC until used for PCR.

2.6.2. DNA purity and concentration: The concentration and purity of genomic DNA were measured using a NanoDrop 2000 Spectrophotometer (Thermo scientific, USA). The Spectrophotometer calculated the concentration of DNA based on the 260/280 absorbance ratio. And a ratio of 1.8 – 2.0 for DNA generally accepted as pure.

2.6.3. Detection of ESBL genes by PCR: Molecular detection of ESBL producing isolates from all collected high vaginal swabs was carried out by PCR. Three specific oligonucleotide primers (Humanizing Genomics macrogen, South Korea) for three genes were used in this study as shown in table (1).

Table 1. PCR primers used for the detection of ESBL genes

Genes	Oligonucleotide sequence (3`- 5`)	Size of amplic ons	Ref.
bla- TEM	F:CAGCGGTAAGATCCTTGAGA	643	Ensor *et al.* (2009)
	R:ACTCCCCGTCGTGTAGATAA		
bla- SHV	F:GGCCGCGTAGGCATGATAGA	714	
	R:CCCGGCGATTTGCTGATTTC		
bla- CTX-M	F:AACCGTCACGCTGTTGTTAG	766	
	R:TTGAGGCGTGGTGAAGTAAG		

The PCR amplification reaction was performed in a final volume of 25 µl; each reaction of PCR contain 1 µl primers (forward and reverse) at a final concentration of 10 pmol/µl each; 12.5 µl of deoxy-ribonucleotide master mix (Promega, Germany); 1 µl of extracted DNA at a final concentration of 50ng/ µl; and 9.5 µl of nuclease free water. The amplification was carried out in (Applied Biosystems™ Veriti™ 96-Well Thermal Cycler, USA), and the cycling conditions of the PCR were illustrated in table (2).

Table 2. PCR conditions of *bla* genes used in this study.

Gene	Initial denaturation	Number of cycles (30)			Final extension	Reference
		Denaturation	Annealing	Extension		
bla_{SHV}	95 ºC /5min.	94 ºC /30sec.	52 ºC /45sec.	72 ºC /45sec.	72 ºC /7min.	Ensor *et al.* (2009)
bla_{TEM}	95 ºC /5min.	94 ºC /30sec.	55 ºC /60sec.	72 ºC /45sec.	72 ºC /7min.	
bla_{CTX-M}	95 ºC /5min.	94 ºC /30sec.	57 ºC /45sec.	72 ºC /45sec.	72 ºC /7min.	

2.6.4. Agarose gel electrophoresis: Amplified PCR products were separated electrophoretically using 2% (w/v) of agarose in (1X) TBE buffer (Promega, USA) with 45V electrical power for 15 minutes before raising to 85 V for 60 minutes. A GelRed stain (Olerup SSP, Sweden) was applied to make the DNA bands visible under UV light of wavelength 365 nm (G: BOX SYNGENE, UK). The band sizes were estimated by comparison to the bands of 100bp–1500bp DNA ladder (GeNet Bio, South Korea).

2.7. Statistical analysis

The results were analyzed statistically by SPSS software version 24 and Microsoft Excel (2013) by using the test of chi-square. The probability value (P-value) less than 0.05 was considered statistically significant. While P<0.01 was considered to be highly significant.

3. RESULTS

3.1. Isolated pathogens

During the current study period, a total of 100 HVS were obtained from pregnant-women patients with symptomatic genital tract infections, the most frequent symptoms reported was abnormal vaginal discharge 18(58.1%), with less frequent 13 (41.9%) cases of women suffering from other symptoms, and mainly of those symptoms also reported with abnormal vaginal discharge, as shown in table (3). About 88(88%) cases were confirmed as positive culture, while 12(12%) samples diagnosed as a negative culture. Among the positive samples, 80(90.9%) were positive for bacterial isolates and 8(9.1%) showed the growth of *Candida species.* No anaerobic bacteria were detected. According to the age groups of cases, the participants ages ranged from (18-45) years old and the mean age was (31.8 ± 6.6) with the minimum and maximum ages being 18 and 45 years old respectively. Vaginal infection was detected in the highest rate 51.6% in the age group of (26-35) years, followed by 32.3% at the age of (36-45) years, and 16.1% at age (18-25). Significant (P < 0.05) relationship was found between vaginal infection and the age group of cases.

The present study is based on the bacterial vaginitis, accordingly, the result revealed that the overall prevalence of bacterial isolates was 80(90.9%), 31(38.8%) were gram negative bacteria, and those with the highest frequency were *Kl. pneumoniae* 17(54.8 %), *E.coli* 11 (35.5%), other gram negative bacteria of lower prevalence were *Acinetobacter spp., Kl. oxytoca,* and *P. aeruginosa* was recorded in 3.2% of cases. as shown in figure (1).

Table 3. Distribution of important symptoms of pregnant patients with vaginal infection

Symptoms and Signs	pregnant patients	
	No.	%
Abnormal vaginal discharge	18	58.1%
Bleeding	3	9.7%
Lower abdominal pain + Abnormal vaginal discharge	4	12.9%
Lower abdominal pain + itching + Abnormal vaginal discharge	2	6.5%
Lower abdominal pain + Burning + Abnormal vaginal discharge	2	6.5%
Abnormal vaginal discharge +Itching	2	6.5%
Total	31	100%

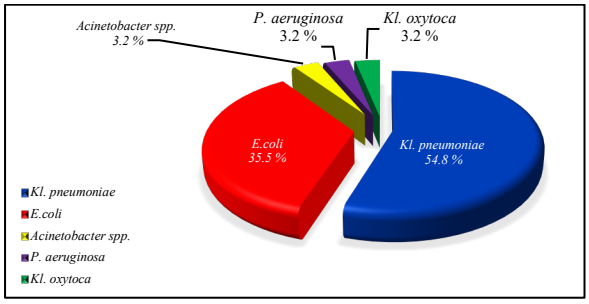

Figure 1. Distribution of bacterial isolates from pregnant women patients with genital tract infection

3.2. Antimicrobial susceptibility testing

Thirty one bacterial isolates were examined to confirm their susceptibility to seventeen antimicrobial agents representing different groups. The reported results in figure (2) showed that all gram negative isolates were 100% resistant to Amoxicillin clavulanic acid and Ampicillin except *Kl. pneumoniae* were 88.2% resistant to Amoxicillin clavulanic acid. On the other hand, the same figure indicated variable resistance properties between isolates versus other antibiotics used, as follows: the clinical isolates of *Acinetobacter spp.* and *P. aeruginosa* were frequently found to be the highest resistant 100% to Ertapenem, Ceftriaxone, Cefuroxime, Ceftazidime, Amoxicillin clavulanic acid, Ampicillin and Trimethoprim. Regarding *Kl. oxytoca* isolate showed highest resistance 100% to Ceftazidime, Amoxicillin clavulanic acid, Ampicillin, Piperacillin, Piperacillin-tazobactam and Trimethoprim. In addition, most isolates of *E.coli* revealed a high level of resistance 100% to Ampicillin and Amoxicillin clavulanic acid; followed by 81.8% to Piperacillin; 63.6% to Ceftriaxone, Cefuroxime and Trimethoprim; 54.5% to Ceftazidime; 45.5% to Amikacin; with less resistance 36.4% to Cefepime and Aztreonam; 27.3% to Gentamicin and Netilmicin; and 18.2% to Ciprofloxacin. While *Kl. pneumoniae* exhibit high resistant 100% to Ampicillin; 88.2% to Amoxicillin clavulanic acid; 70.6% to Piperacillin; 64.7% to Gentamicin; 58.8% to Cefuroxime, Ceftriaxone, Ceftazidime and Cefepim; 52.9% to Trimethoprim; 47.1% to Aztreonam and Ciprofloxacin; with low level of

resistance 35.3% to Amikacin and Netilmicin; 29.4% to Piperacillin-tazobactam; 17.6% to Ertapenem; and 11.8% to Imipenem and Meropenem.

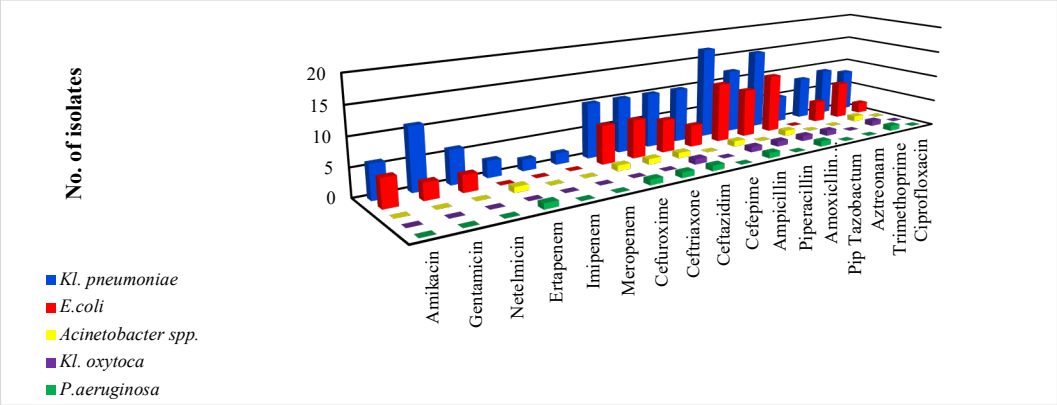

Figure 2. Antibiotic resistance pattern of gram-negative bacterial isolates from women patients with GTI.

The results of the present study revealed that the efficacy of Imipenem, and Meropenem to most species of gram negative bacteria was 100% susceptible as shown in figure(3), except for *Kl. pneumoniae* isolates which were susceptible by 88.2%. *P. aeruginosa* and *Acinetobacter spp.* isolates revealed a high level of sensitivity 100% to Amikacin ,Gentamicin, Netilmicin, Imipenem, Meropenem, Cefepime, Aztreonam, Piperacillin, Piperacillin-tazobactam and Ciprofloxacin. While *Kl. oxytoca* showed 100% susceptible to Amikacin, Gentamicin, Netilmicin, Imipenem, Meropenem, Ertapenem, Ceftriaxone, Cefuroxime, Cefepime, Ciprofloxacin and Aztreonam. *E. coli* isolates exhibit high level of sensitivity 100% to Ertapenem, Imipenem, Meropenem and Piperacillin-tazobactam; followed by 81.8% to Ciprofloxacin; 72.7% to Amikacin and Netilmicin; 63.6% to Cefepime and Aztreonam; 54.5% to Amikacin; 45.5% to Ceftazidime; with less susceptible 36.4% to Cefuroxime, Ceftriaxone and Trimethoprim; and 18.2% to Piperacillin. *Kl. pneumoniae* isolates were 88.2% sensitive to Imipenem and Meropenem; 82.4% to Ertapenem; 70.6% to Piperacillin-tazobactam; 64.7% to Netilmicin and Amikacin; moderately 52.9% to Aztreonam and Ciprofloxacin; 47.1% to Trimethoprim; 41.2%to Cefepime, Cefuroxime, Ceftazidime and Ceftriaxone; with low level of sensitivity 35.3% ; 29.4%; 11.8% to Gentamicin, Piperacillin, and Amoxicillin clavulanic acid respectively.

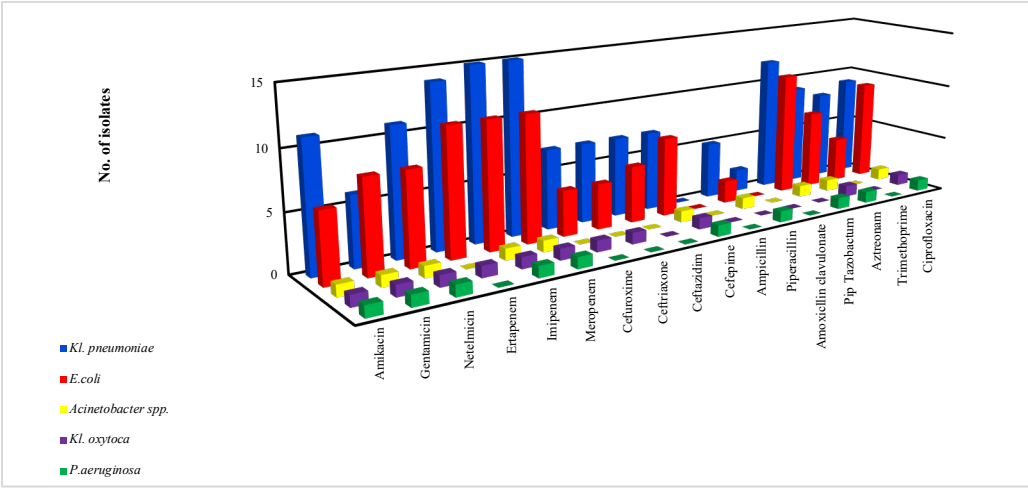

Figure 3. Antibiotic sensitivity pattern of gram-negative bacterial isolates from women patients with GTI.

3.3. Incidence of multidrug resistant pattern in all bacterial isolates studied

A multidrug resistant profile has been used to recognize MDR, XDR and PDR in all bacterial isolates studied, which is determined as follow: Out of total 31 bacterial isolates, 54.8% were MDR, 29% were XDR and PDR were not detected, as illustrated in table (4). Among 17 isolates of *Kl.pneumoniae* 7(41.2%) were multidrug resistant and extensively drug resistant, out of 11 *E.coli* isolates 7(63.6%) were MDR and 2(18.2%) were XDR. The overall rate ofmultidrug resistant among all isolates (*Acinetobacter spp., P. aeruginosa and Kl. oxytoca*) were 100%, with no XDR.

Table 4. Frequency of MDR and XDR of gram negative bacterial isolates for selected antimicrobial classes.

Bacterial isolates(n)	Types of resistance	
	MDR	XDR
Kl. pneumoniae (17)	7(41.2%)	7(41.2%)
E.coli (11)	7(63.6%)	2(18.2%)
Acinetobacter spp. (1)	1(100%)	0
P. aeruginosa (1)	1(100%)	0
Kl. oxytoca(1)	1(100%)	0
Total 31	17 (54.8%)	9 (29%)

3.4. Screening for ESBL producers

Thirty one gram negative bacterial isolates obtained from pregnant patients with bacterial vaginitis were studied phenotypically and genotypically for ESBL production; the results revealed that 19(61.3%) isolates were ESBLs producers and 12(38.7%) were non ESBLs producers. No significant relationship (P<0.05) was found between ESBL producer and non ESBL producer with the frequency of bacterial vaginitis. Beta-lactamase production were observed in 9(81.8%), and 10(58.8%) of *E.coli* and *Kl. pneumoniae* isolates respectively.

3.5. Antimicrobial Susceptibility Pattern of ESBL producing isolates

The resistance profile for studied clinical isolates revealed that the drug resistance rate was higher in ESBL producers that in non ESBL producers as shown in table (5). It was found that the highly resistant ESBL isolates were Ampicillin and Amoxicillin clavulanic acid 100%, Piperacillin 94.7%; while non-ESBL-producing isolates showed less resistance to the same antibiotics. Additionally, ESBL producing isolates demonstrated high sensitivity 94.7%, 89.5% and 84.2% for Imipenem, Meropenem and Ertapenem respectively. A significant difference (P<0.01) was found in the resistance pattern with Cephalosporins between ESBL and non-ESBL isolates; resistance rate to Cefuroxime and Ceftriaxone were 89.5%, and 84.2% and 73.7% to Ceftazidime and Cefepime respectively; while non ESBL producer isolates showed higher sensitivity to these antibiotics. Moreover, high resistance was recorded toward other antimicrobial agents tested (P<0.01): Aztreonam 63.2%, Amikacin 57.9% and Netilmicin 47.4%.

Table 5. Antibiotic Susceptibility pattern of ESBL and non-ESBL producing isolates

Antibiotics	ESBL positive		ESBL negative		P-value
	Resistance	Sensitive	Resistance	Sensitive	
Amikacin	11(57.9%)	8(42.1%)	0(0%)	12(100%)	P<0.01
Gentamicin	10(52.6%)	9(47.4%)	4(33.3%)	8(66.7%)	P>0.05
Netilmicin	9(47.4%)	10(52.6%)	0(0%)	12(100%)	P<0.01
Ertapenem	3(15.8 %)	16(84.2%)	1(8.3%)	11(91.7%)	P>0.05
Imipenem	1(5.3 %)	18(94.7%)	0(0%)	12(100%)	P>0.05
Meropenem	2(10.5%)	17(89.5%)	0(0%)	12(100%)	P>0.05
Cefuroxime	17(89.5%)	2(10.5%)	2(16.7%)	10(83.3%)	P<0.01
Ceftriaxone	17(89.5%)	2(10.5%)	2(16.7%)	10(83.3%)	P<0.01
Ceftazidime	16(84.2%)	3(15.8 %)	3(25 %)	9(75%)	P<0.01
Cefepime	14(73.7%)	5(26.3%)	0(0%)	12(100%)	P<0.01
Ampicillin	19(100%)	0(0%)	12(100%)	0(0%)	P>0.05
Piperacillin	18(94.7%)	1(5.3 %)	4(33.3%)	8(66.7%)	P<0.01
Amoxicillin clavulanic acid	19(100%)	0(0%)	10(83.3%)	2(16.7%)	P>0.05
Piperacillin – tazobactam	5(26.3%)	14(73.7%)	1(8.3%)	11(91.7%)	P>0.05
Aztreonam	12(63.2%)	7(36.8%)	0(0%)	12(100%)	P<0.01
Trimethoprim	11(57.9%)	8(42.1%)	8(66.7%)	4(33.3%)	P>0.05
Ciprofloxacin	6(31.6%)	13(68.4%)	4(33.3%)	8(66.7%)	P>0.05

3.6 Phenotypic results for ESBL production

Phenotypically,p ESBLs production was confirmed by both double disk synergy test and ESBL CHROMagar methods as shown in (Figure 4 and Figure 5) respectively. Among gram negative isolates, 15(48.4%) and 18(58.1%) were detected as ESBLs producers by double disk synergy test and CHROMagar respectively.

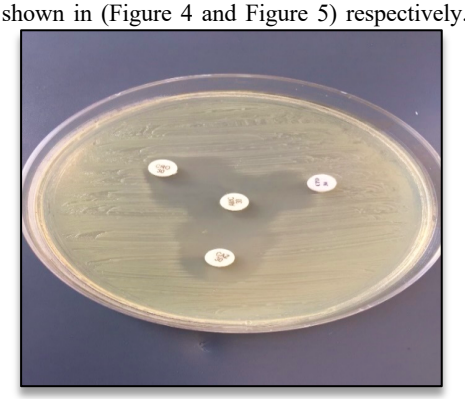

Figure 4. positive double disk synergy test for ESBL production showing synergy between 3rd generation cephalosporin (Ceftazidime, Ceftriaxone and Cefotaxime) and Amoxicillin clavulanic acid disks

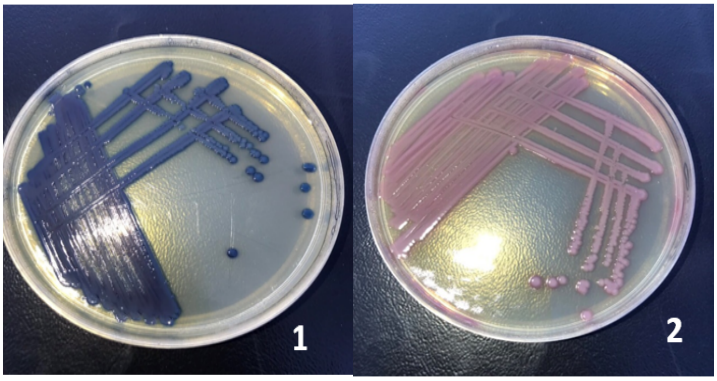

Figure 5. ESBL producers colonies on CHROMagar : 1-*Kl. pneumoniae* and 2- *E.coli*

3.7 Molecular characterization of *bla* genes

Polymerase Chain Reaction was used to detect the Beta-lactamase genes (CTXM , SHV and TEM) in all gram negative bacterial isolates harbored ESBL, using specific oligonucleotide primers, as shown in (Figure 6, Figure 7 and Figure 8). (The results indicated that, out of 19 isolates, there were 14(73.7%) positive for $bla_{\text{CTX-M}}$ gene, 11(57.9%) were positive for bla_{SHV} gene, and 10 (52.6%) were positive for bla_{TEM}. Also, the results illustrated that CTX-M gene was most predominant detected gene within isolates of ESBLs-producing *E.coli* 9(100%); while TEM gene ,and SHV types were observed in 4(44.4%) and 2(22.2%) respectively. On the other hand, 9(90%) of ESBL-*Kl. pneumoniae* isolates have carried SHV gene; while TEM type and CTX-M genes were reported in 6(60%) and 5(50%) respectively.

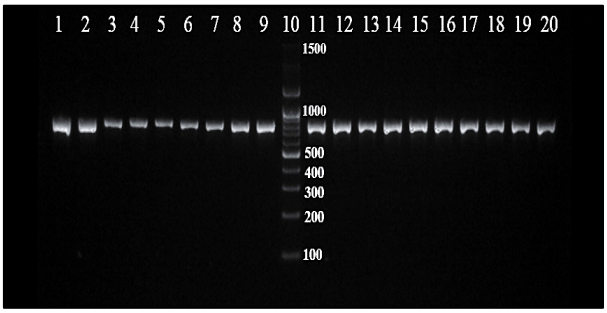

Figure 6. Result of PCR amplified of $bla_{\text{CTX-M}}$ gene, using 2% agarose gel electrophoresis, show positive results at 766 bp; Lane 1 to 9 contain ESBL producing *E.coli*; lane 10 DNA ladder (1500 bp); and ESBL producing *Kl. pneumoniae* lane 11 to 20.

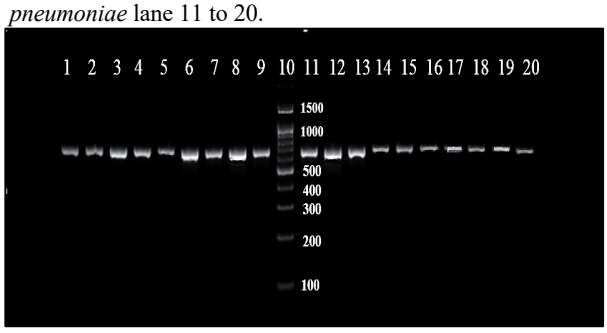

Figure 7. Result of PCR amplified of bla_{TEM} gene, using 2% agarose gel electrophoresis, show positive results at 643 bp; Lane 1 to 9 represent ESBL producing *E.coli* ;lane 10 DNA ladder (1500 bp).lane 11 to 20 contain ESBL producing *Kl. pneumoniae*.

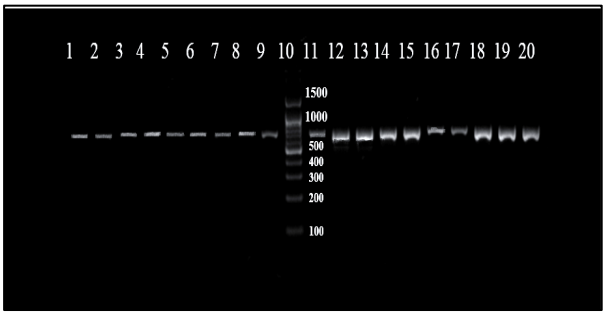

Figure 8. Result of PCR amplified of bla_{SHV} gene, using 2% agarose gel electrophoresis, show positive results at 714 bp; Lane 1 to 9 contain ESBL producing *E.coli* ;lane 10 DNA ladder (1500-bp); and ESBL producing *Kl. pneumoniae* lane 11 to 20.

Table (6) revealed that the main ESBL genotype patterns distribution between isolates were present alone or in combination with each other. It has been found that 3 (30%) among *Kl.pneumoniae* isolates carried the three ESBLs genes (TEM+SHV+CTXM); bla_{SHV}, both TEM+SHV and SHV+CTX-M types were observed in 2(20%) isolates, and one isolate (10%) had only TEM gene. On the other hand, among 4(44.4%) ESBL producing *E.coli* harboring only CTXM gene; followed by genotype combination TEM+CTX-M 3(33.3%); while one isolate (11.1%) harbored SHV+CTXM and carried three ESBL genes (TEM+SHV+CTXM).

Table 6. Distribution of *bla* gene in ESBL producing isolates.

Patterns of ESBL genotype	*Kl. pneumoniae*	*E.coli*	Total
TEM only	10%	0	5.3%
SHV only	20%	0	10.5%
CTX-M only	0	44.4%	21.1%
TEM + SHV	20%	0	10.5%
TEM + CTX-M	0	33.3%	15.8%
SHV + CTX-M	20%	11.1%	15.8%
TEM+SHV+ CTX-M	30%	11.1%	21.1%

4. DISCUSSION

Vaginal infection in pregnant women is a health problem that results in complication and serious medical consequences. The prevalence rate of vaginal infection was 88% among symptomatic pregnant women who were enrolled in our study. Our results were lower compared to a study conducted by Ahmad and Ali (Ahmad and Ali , 2015) whose results yielded 95%; while Divya and Karthika, 2015; Ravishankar and Prakash, 2017; Razzak *et al*., 2011 and Pal *et al.*, 2017 which reported a lower vaginitis prevalence (51%, 50.4%, 29.5% and 29.3% respectively) among pregnant women. These differences may be attributed to the variation in study techniques for isolation and identification of the causative agents of vaginal infection, species prevalence differs among the different geographical region and difference on study participant. In this study, the high rate of positive cultures of vaginal infection may be because the pregnancy is usually accompanied by many immunological, physiological and hormonal changes which make pregnant women vulnerable to many infections. Negative bacterial cultures were observed in 12% high vaginal swabs in the present study, which may be due to the possibility of chlamydia, viruses and other agents as pathogens of vaginitis that need special techniques for their detection. Table (1) indicates that the high percentage of important symptoms relevant to vaginal infection was abnormal vaginal discharge (58.1%). Similar results have been obtained by Pal *et al.* (2017) from India (58.5%), and Ahmad and Ali (2015) from Iraq in a rate of (56.8%).This could be attributed to the fact that the dominant normal microbial vaginal flora was replaced by many other opportunistic pathogens among women with abnormal discharge. Regarding the age pattern of vaginal infection, the results of the present study indicated that young sexually active women in the age group 26-35 had a high incidence of vaginal infection (51.6 %). Statistical analysis revealed that the incidence of infections at the age group (26-35) years significantly ($p < 0.05$) higher in compare to the other age groups. This was in concordance with a study conducted by Ahmad and Ali (2015). Another study by Sangeetha *et al*. (2015) showed that these infections were prevalent (30%) in the 26-30 age group, followed by (26.08%) in the 31-35 age group, and (20.8%) in the 21-25 age group. Pal *et al.* in 2017 reported a high frequency of infection (65.3%) at 25-30 years followed by 31-35 years (60%). Lamichhane *et al.* (2014) detected the highest percentage (68.5%) in age group 20-29 years and (1.09%) aged 40 and above. The different infection patterns in this study may be due to prevailing conditions such as educational level, health awareness, health

care and it's availability in every country. The results proved that 38.8% of gram negative bacteria detected in high vaginal swabs collected from symptomatic pregnant women, this result is in agreement with earlier studies performed by Razzak *et al*. (2011) and Divya and Karthika (2015), while (Khamees, 2012; Ravishankar and Prakash, 2017; Pal *et al.*, 2017) reported a rate 59.2%, 57.5% and 56.6% respectively in pregnant women. The reason for this variation is attributed to samples and population studied as well as to virulence factors of opportunistic bacteria and their role in pathogenicity. Among the isolated gram negative bacteria from women with vaginitis, *Kl. pneumoniae* (54.8%) was the predominant pathogen followed by *E.coli* (35.5%). The isolation of those fecal pathogens is due to the unique anatomical feature of the female genitourinary tract with shorter urethra and a more proximal location of urethra meatus to the anus (Donders *et al.,* 2002); moreover, individuals were at risk of infection due to low socioeconomic status related to poor hygiene. The antimicrobial susceptibility patterns for all bacterial isolates in this study were completely variable. Carbapenems (Imipenem and Meropenem) and Piperacillin-tazobactam were the most effective antibiotics against 93.5% and 80.6% of all studied isolates, meanwhile the majority of gram negative bacterial isolates showed high level of resistance (93.5%-100%) to Amoxicillin clavulanic acid and Ampicillin in this study. Different studies reported different susceptibility patterns; similar findings have been obtained by Ahmad and Ali (2014) from Iraq who reported that all gram-negative bacterial isolates were susceptible to Meropenem, but 6.8% were sensitive to Imipenem, on the other hand 89% of isolates were resistance to Ampicillin and 56.2% to Amoxicillin clavulanic acid. Most effective antibiotics against *E.coli* and *Kl. pneumoniae* in the present study were Imipenem and Meropenem (100%, 88.2%), Ertapenem (100% -82.4%), Piperacillin-tazobactam (100% - 70.6%). This is in agreement with studies by Tariq *et al.* (2006), and Mumtaz *et al*. (2008) who found that most *E.coli* and *Kl. pneumoniae* isolates were high sensitive to Imipenem and Piperacillin-tazobactam. Tang *et al.* (2020) observed that *E.coli* isolates had high susceptibility (100%) to Ertapenem, Imipenem and Meropenem. Similar results were obtained from Iraq, where Al-Mayahie study reported that all *E.coli* that isolated from pregnant and non-pregnant patients were susceptible to Imipenem, and Meropenem, whereas 100% resistance to Amoxicillin clavulanic acid (Al-Mayahie, 2013). An important reason for this difference attributed to geographical difference between countries and their antibiotics prescription policy. The drug resistant isolates in the current study were common; worryingly high. Overall, 54.8% of bacterial isolates among symptomatic pregnant women in this study were characterized as MDR pathogenic bacteria, 29% isolates were XDR; no PDR were detected. This detection of drug resistant bacterial isolates may limit treatment options. Therefore, the wise use of appropriate antimicrobial agents is recommended. In addition, the current results demonstrate a high level of multidrug resistant isolates which are in accordance with the study done in India where 61.2% of *E.coli* isolates, 60% by *Klebsiella spp.* and also 60% *Acinetobacter spp.* Isolated from high vaginal swabs were found to be MDR (Ravishankar and Prakash, 2017). In another similar study, 52.9% of *E.coli* isolates, 33.3% by *Kl. pneumoniae* isolates from high vaginal swabs were MDR (Lamichhane *et al.*, 2014). The reason for this high rate of MDR among symptomatic females genital tract infection may due to its association with miss use of antibiotics, inappropriate prescription and virulence properties of potential vaginal pathogens that have a high ability to avoid the antimicrobial effects. There is a significant geographical difference in the occurrence of ESBLs worldwide (Coque *et al.*, 2008; Leylabadlo *et al.*, 2017). Overall, the rate of ESBLs producing isolates was(61.3%) in our study, which is higher when compare to the results of other local study conducted by Ahmad and Ali (2014) which found that the rate of isolates producing ESBL from pregnant women was 45%. Other studies from Sudan and Lebanon have reported that the percentage of isolates producing ESBL among symptomatic females with genital tract infection was 24.3% and 19.1% respectively (Gorish, 2019; Gaddar *et al*., 2020). The member of *Enterobacteriaceae* use several resistance mechanisms for avoiding the effects of antimicrobial agents, however, β-lactamases production is the most important and clinically significant resistance mechanism against β-lactam antibiotics (Wax *et al*., 2008). The proportion of ESBL producers was higher in *E.coli (*81.8 %) than *Kl. pneumoniae* (58.8%) in this study. Overall, ESBL-producing isolates revealed high resistance against Ampicillin and Amoxicillin clavulanic acid, while all isolates showed sensitivity towards Carbapenems. According to our results, ESBL-producing isolates showed significantly higher resistance to 3rd and 4th generation Cephalosporins than non ESBL-producing isolates. These results are on line with others studies performed in Iraq (Jabbar, 2013; Al-Mayahie, 2013; Ahmad and Ali, 2014; Mawlood *et al*., 2018). The high prevalence of infection with ESBL may be attributed to widespread resistance to broad spectrum Cephalosporins in our country as a result; it may be the most prominent risk factor for emergency of ESBL producing pathogens (Al-Hilali, 2010). Hence, such studies should be carried on a continuous basis to detect the emergency of the most recent strains in the region by routine screening for β-lactamases production, this indicates that there is a sever need for contentious monitoring system and effective measures to control infection. All positive ESBL-producing isolates were confirmed by PCR-assay for detection of CTX-M, SHV and TEM genes. ESBL genotype $bla_{\text{CTX-M}}$ was the predominant gene (73.7%) followed by bla_{SHV} and bla_{TEM} (57.9% and 52.6%) respectively. This is consistent with the current situation around the world , including most European countries, Latin America and East Asia, where CTX-M type have replaced TEM and SHV types as the predominant ESBL among Enterobacteriaceae (Hawkey, 2008; Livemore, 2012). Previous studies in Iraq and in the neighboring countries have shown that CTX-M gene was a most prevalent *bla*-gene in both *E.coli* and *Kl. Pneumoniae*. (Jabbar, 2013; Al-Mayahie, 2013; Hasan *et al*., 2013). But, studies from Turkey observed that TEM was the predominant type (Oksuz and Gurler, 2009; Bali *et al*., 2010; Dagi, 2015). On the other hand, Mawlood *et al.*, 2018 reported that SHV type ESBL was more frequently found in *Kl. pneumoniae*, and our data confirms this result. This indicates that the prevalence of ESBL genes (CTX-M, SHV and TEM) differ among patients groups, clinical setting and geographical regions. Our results document that among 19 ESBL isolates seven genotypes pattern of ESBL were observed, and the predominant genotype was CTX-M type (21.1%), followed by the combination of the CTX-M gene with TEM and SHV types separately (15.8%). TEM+CTX-M combinations has been observed to be the prevalent genotype in Saudi Arabia, Japan and Lebanon, (Bindayna *et al.*, 2010; Harada *et al.*, 2013; Gaddar *et al*., 2020). On the other hand, another study in Macedonia (Kaftandzieva *et al.*, 2011) showed the predominance of the genotype TEM+SHV combination. The presence of more than one genotype in same strains producing ESBL may be correlated with increased resistance levels. Combination of (TEM+SHV+CTX-M) can lead to Carbapenemes resistance; this is worrying and more serious for community (Manoharan *et al.*, 2011). So, this is considered as one of the more problematic aspect in treating infections caused by gram negative bacteria. Therefore, screening for ESBL production should be performed routinely in every clinical diagnostic laboratory to guide clinicians in the appropriate selection of antibiotics.

5. CONCLUSION

Bacterial vaginitis was observed to be more common among young, sexually active female in the age group (26-35) years, and the incidence of infection at this age group was significantly higher compared to other age group. This study gave strong indication about the different microorganism, present in women who complain of abnormal discharge. The most common ESBL producing isolates were *E.coli* and *Kl. pneumoniae*. Drug resistant isolates in this study were common, worryingly high and it may limit treatment options available, hence, efforts to isolate microorganism and determine the susceptibility pattern should improve the treatment of vaginal infection rather than usual trend of empirical treatment. This study demonstrated the presence of a high level of $bla_{\text{CTX-M}}$ gene among ESBL isolates which are wide spread in this area.

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