

Molecular Screening of Extended Spectrum β -lactamases in *Klebsiella pneumoniae* isolated from Clinical Sources

Sawsan Abdulhameed Jassim¹, Muthanna Hamid Hassan²

^{1,2}University of Anbar, College of science, Department of biology

Email: Susan20ms@gmail.com

DOI: 10.47750/pnr.2022.13.S06.317

Abstract

Klebsiella pneumoniae is a major pathogen responsible for nosocomial infections in world hospitals. One of the causes for the drug resistance in the *Klebsiella pneumoniae* is the production of ESBLs enzymes. This research documented TEM, SHV, and CTX-M for the first time among *Klebsiella pneumoniae* in Al-Anbar hospitals of Iraq. All studied clinical isolates of *K. pneumoniae* (30) resistant- beta lactam from 100 isolates were collected from different clinical sources such as (burned, wounds, sputum, and urine samples). The susceptibility to different antibiotics was tested by VITEK-2 system. The bla TEM, SHV, and CTX-M genes were detected by conventional PCR and the result showed 30/30 (100%) strains harbored these genes. This result showed the coexistence of these genes in one strain of *K. pneumoniae*, while indicated widespread ESBLs in Anbar, Iraq.

Keywords: TEM, SHV, CTX-M, ESBLs, *Klebsiella pneumoniae*.

INTRODUCTION

Klebsiella pneumoniae is a significant Enterobacteriaceae considered as one of the opportunistic pathogens causing broad spectra of diseases and showing increasingly frequent acquisition of resistance to antibiotics, specifically the extended-spectrum β -lactamase (ESBL)-producing strains (1). Shiri et al. conducted that *Klebsiella pneumoniae* accounts for about one-third of all Gram-negative infections such as urinary tract infections, cystitis, pneumonia, surgical wound infections, endocarditis and septicemia (2). Extended-spectrum β -lactamases (ESBLs) are enzymes that hydrolyze penicillins, expanded-spectrum cephalosporins, and monobactams and are commonly inhibited by β -lactamase inhibitors such as clavulanic acid, sulbactam, tazobactam, and natural products such as medicinal herbs (3). Nowadays, More than 600 ESBL variants have been described and the majority of them belong to the SHV, TEM and CTX-M families (4).

K. pneumoniae infections are difficult to treat, particularly because of the pathogen's high endogenous antibiotic resistance. For example, *K. pneumoniae* is intrinsically resistant to cephalosporins, owing to the presence of ESBLs (SHV) encoding genes in its chromosomal genome (5). In addition it was incriminated for the appearance of multidrug resistant (MDR) strains against third generation cephalosporins, fluoroquinolones, carbapenem and aminoglycosides (6). The correlation between its wide ecological range and its ability to carry multidrug resistance genes makes of *K. pneumoniae* a good candidate for dissemination and horizontal gene transfer among the Gram-negative species (7). Horizontal transfer of antibiotic resistance genes between different species of bacteria is facilitated by mobile DNA elements such as transposons and plasmids. In the recent years, a substantial portion of the resistance genes present on the plasmids and transposons of Gram-negative bacilli have been observed to be integrated into DNA elements called integrons (8). This study aimed to detect TEM, SHV, and CTX-M among *Klebsiella pneumoniae* isolates.

Study Design:

One hundred *Klebsiella pneumoniae* isolates were isolated from patients admitted to Al-Ramadi Teaching Hospital , Al-Fallujah and outpatients from Private Clinics. All samples (257) were collected during the period from August to November 2021. The patients were of different genders. Full informative history was taken from the patient or his parents or relatives. The study specimens were obtained from midstream urinary tract infections and other infections, including burns, blood , sputum, and wounds.

Ethics and approval committee:

All study techniques that involved patients were approved by the Ethical Approval Committee, University of Anbar, Ramadi, Iraq (approval number 73, 27-6, 2022). Informed, written consent was provided by all patients participating in the study.

Identification of *Klebsiella pneumoniae*:

All isolates were cultivated on MacConky agar, and blood agar, which were used for the primary morphological properties of bacterial growth under a microscope , biochemical diagnosis , automated diagnosis using VITEK-2 compact system.

Antibiotic susceptibility test:

Antimicrobial susceptibility was performed test according to Kirby–Bauer method (9) which was modified by World Health Organization (10) and using AST card by automated Vitek-2 system. As shown in figure 1.

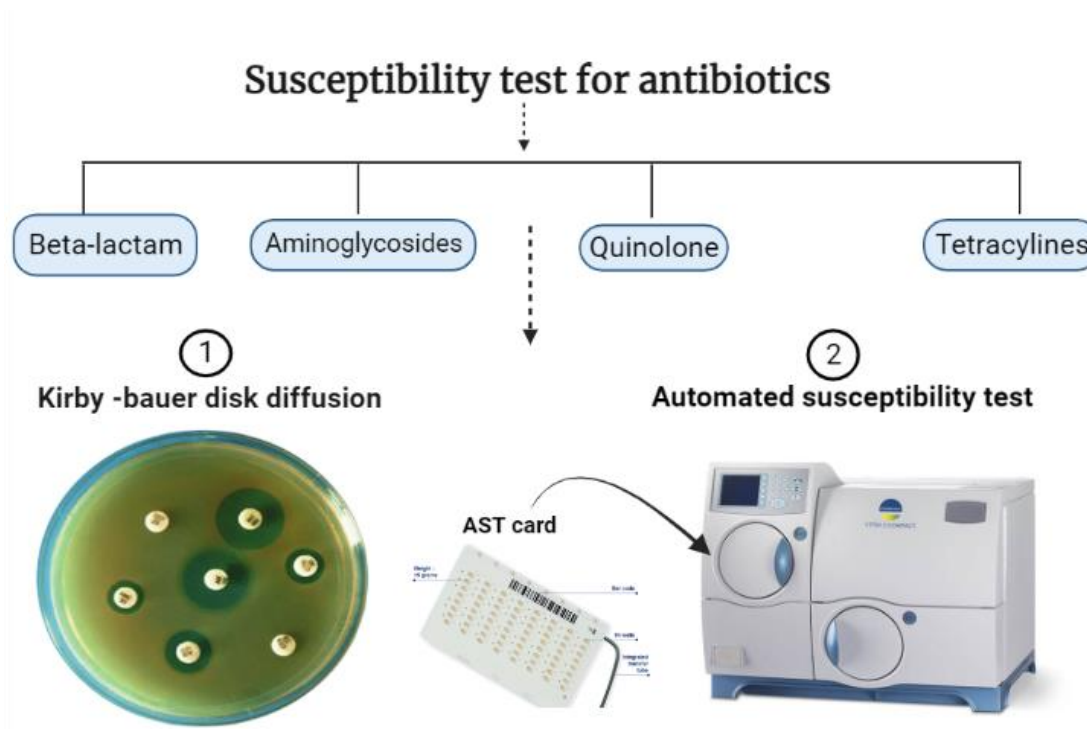


Figure 1 : Schematic diagram illustrating susceptibility test methods.

Molecular study:

DNA extraction:

Genomic DNA of *Klebsiella pneumoniae* isolates were extracted from different sources using Presto™ Mini gDNA Bacteria Kit, purification depending on the instruction of the manufacturing company (Geneaid, Taiwan). The bacterial DNA was extracted according to DNA - Extraction (Genomic) Kit provided by Geneaid Biotech Ltd. Company (Taiwan).

Determination of DNA concentration and purity using spectrophotometry method:

The DNA concentration of samples was estimated by using Nano drop Spectrophotometer, as follows :

1 µl of TE buffer was used as blanking .

1 µl of the extracted DNA was put in the Nanodrop instrument to detect concentration in ng/µl, and purity was detected by the ratio of absorbance at wavelength 260/280 nm

Primer dilution:

All primers were supplied by bioneer Company as a lyophilized product of different picomols concentrations and resuspension using nuclease free water to reach a final concentration for 10 picomols /µl of suspension.

Detection of ESBLs genes :

The PCR reactions were prepared in a total volume of 25 µL and amplification was performed in a thermocycler (Bioapplied cyclor®, USA) as follows

Detection of bla SHV and bla TEM : initial denaturation 94°C for 5 min. 35 cycles of denaturation 94°C for 40 s, annealing 60°C for 40 s , extension 72°C for 1 min with a final elongation step at 72°C for 7 min and 4°C∞.

Detection of bla CTX-M: initial denaturation 96°C for 10 min. 35 cycles of denaturation 94°C for 1 min, annealing 58°C for 1 min and elongation 72°C for 1 min with a final elongation step at 72°C for 10 min , and 4°C∞.

Reaction of PCR :

For Polymerase chain reaction (PCR) analysis of ESBLs and integrons genes, bacterial DNA was extracted from thirty isolates of *Klebsiella pneumoniae* using DNA extraction kits . As shown in table 1.

Table 1: The original PCR reagents and final concentrations of the protocol.

Component	Volume
Go Taq Green Master Mix 2x	12.5 µl
Forward primer	1 µl
Reverse primer	1 µl
DNA template	1 µl (60 ng)
Nuclease free water	9.5 µl
Final volume	25 µl

Agarose gel electrophoresis:-

After PCR amplification, agarose gel electrophoresis was adopted to confirm the presence of amplification. PCR was completely dependable on the extracted DNA criteria.

Results and discussion

Isolation of *Klebsiella pneumoniae* :

A total of (257) samples were collected from August to November of 2021 from the Al-Ramadi Teaching Hospital, Maternity and Children Hospital patients, and from outpatients visiting private clinics in this city during this period. These samples included: burns and wounds, urine, sputum and blood samples. Samples were collected using sterile cotton swabs, while the mid-stream urine and sputum samples were collected in sterile plastic containers.

In the current study, 100/257(38.9105%) samples were identified as *Klebsiella pneumoniae* and other samples were identified as different bacterial species with ratio 61.0895% . Numerous studies reported that *K. pneumoniae* has such percentages as 4.03%, 17.36%, and 32.48%(11);(12);(13). These differences in the mean prevalence rates among various studies could be related to differences in geographical location and hygienic practices of the population (14);(15). *K. pneumoniae* is the most prevalent cause of nosocomial infections and is considered an opportunistic pathogen due to the difficulty and misclassification in the detection of this bacterium in the laboratory (16).

Identification of *Klebsiella pneumoniae*

As shown in table 4-1, results of biochemical test that bacteria identified as *K. pneumoniae* . The bacterial strain was confirmed using the biochemical reactions by Bergey's Manual of Systemic bacteriology and Finegold and Marti (17) .Specific biochemical tests were performed for additional verification, including gram-negative rods. One-hundred specimens show a positive outcome for catalase and negative for oxidase and gram stain. The IMViC tests are a group of individual tests used in microbiology lab - biotechnology department testing to identify an organism . The term "IMViC" is an acronym for each of these tests. "I" is for indole test; "M" is for methyl red test; "V" is for Voges-Proskauer test, and "C" is for citrate test. The lowercase "i" is merely for "in" as the Citrate test requires coliform samples to be placed "in Citrate".

Table 4-1: Biochemical tests for *Klebsiella pneumoniae* bacteria isolates

Test	Characteristics	<i>Klebsiella pneumoniae</i>
	Catalase	+ve
	Oxidase	-ve
	large, mucoid, pink, and lactose fermenter colonies	MacConkey agar
	γ -hemolytic grey-white, and mucoid colonies	Blood agar
IMViC	Indole	-ve
	Methyl Red	-ve
	Citrate	+ve
	Urease	+ve
	VP	+ve

Automated identification :

The VITEK 2 system combines several advantages that may be of clinical interest for routine testing of gram-negative rods isolated from clinical samples: rapid identification (3hr), a simple methodology, a high level of automation, and taxonomically updated databases. In our study the VITEK 2 system identified all *Klebsiella pneumoniae* with high level probability ranged 94% to 99% with a time 3.5 hr , While *Escherichia coli* ATCC25922 (Appendix5)isolates identifies with high level 99% probability at 7hr . Results from previous studies indicate that the VITEK 2 system correctly identified 85.3 to 100% of *P. aeruginosa* strains (18). The VITEK 2 system identified a significant number of fermenting gram-negative rods within 3 h, which may be clinically relevant, because rapid reporting of microbiology results to physicians has been shown to significantly reduce the mortality rate and to favor earlier initiation of appropriate antimicrobial therapy, a shorter hospital stay, and a lower average variable cost per patient (19) , (20).

Antibiotics Susceptibility Test:

The antibiotic sensitivity test was chosen in the present study because it is the primary and basic test in determining *Klebsiella pneumoniae* isolates producing β -ESBLs. On the other hand, Extended spectrum β -lactamase (ESBL)-producing *Klebsiella pneumoniae* poses unique challenges to clinical microbiologists, clinicians, infection control professionals and antibacterial-discovery scientists. ESBLs are enzymes capable of hydrolysing many antibiotics such as penicillins, broad-spectrum cephalosporins and monobactams. ESBLs are often located on plasmids that are transferable from strain to strain and between bacterial species.

One hundred isolates of *K. pneumoniae* from the wound, burn, sputum, blood and urinary tract infection patients were verified for antibiotic sensitivity by Kirby Bauer disks diffusion method and AST card based on the recommendation of (21) appendix 2, its Susceptibility was tested to twelve antimicrobials including Ampicillin, Piperacillin-Tazobactam, Amox-clav, Ceftriaxon, Cefotaxime, Ceftazidime, Cefipime, Meropenem, Imipenem, Amikacin, Ciprofloxacin, Levofloxacin, and Tigecycline. This study showed that there was a high level of antimicrobial resistance among *K. pneumoniae* isolates. Of the 100 *K. pneumoniae* isolates tested in this study demonstrated resistance to antimicrobials such as ampicillin 90%, piperacillin-tazobactam 85%, amox-clav 95%, ceftriaxon 77%, cefotaxime 74%, ceftazidime 69%, cefipime 47%, meropenem 33%, imipenem 31%, amikacin 30%, ciprofloxacin 31%, levofloxacin 27%, tigecycline 10% figure 2. The results revealed variations of isolates resistant to all antibiotic used in the present study. The increase of bacterial resistance to several antibiotics considered is as a tremendous therapeutic difficulty.

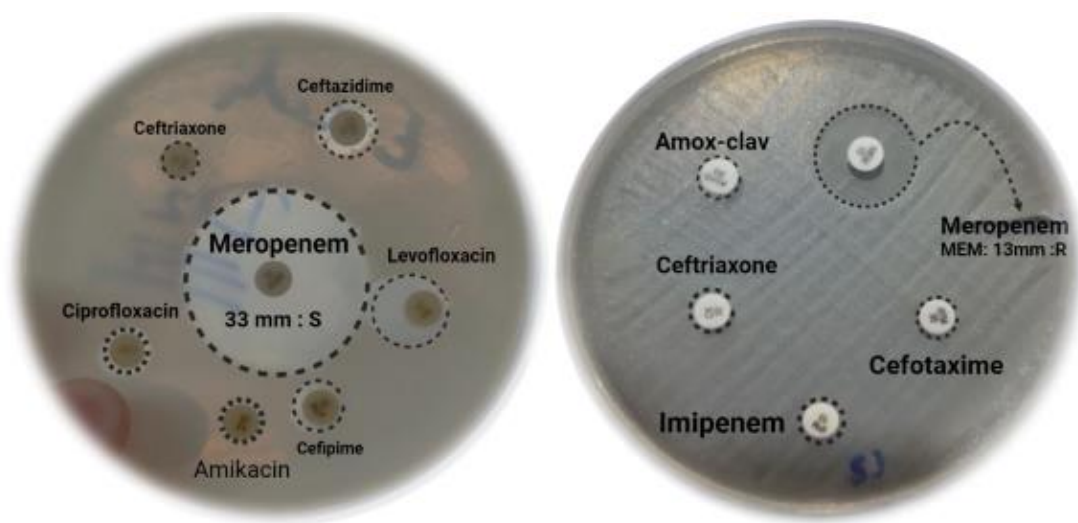


Figure (2): Kirby-Bauer Disk Diffusion Susceptibility Test for antibiotics in *K. pneumoniae*

Classification of isolates based on their drug resistance pattern:

As shown in Figure 3, among 30 bacterial isolates, 22 (73.33%), 6 (20%), and 2(6.667%) were MDR, XDR, and PDR respectively.

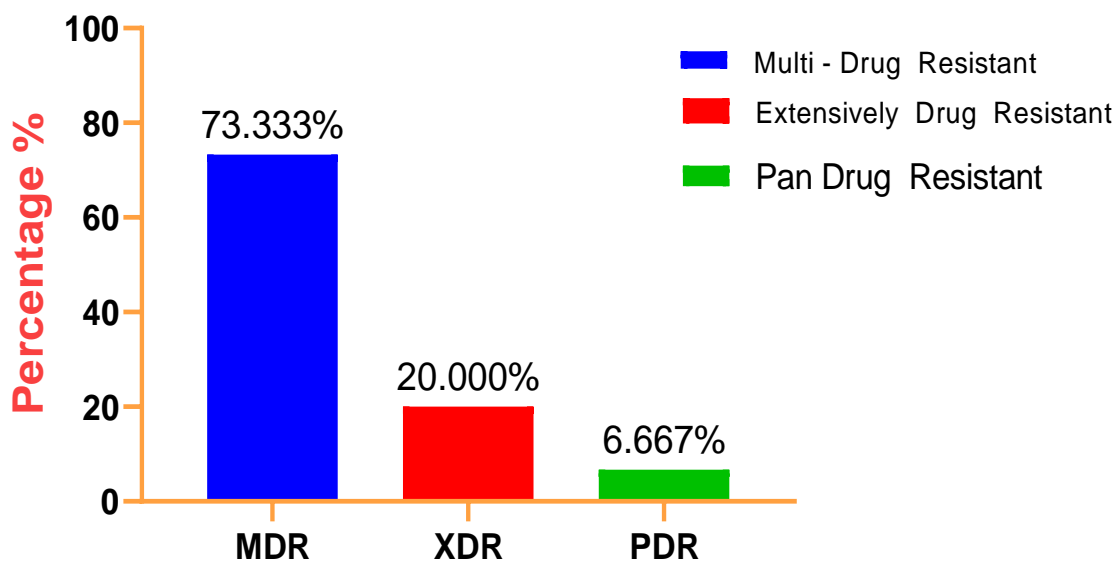


Figure (3): Distribution of PDR , XDR , and MDR among K. pneumoniae in AL-Anbar province.

MDR K. pneumoniae has become an important challenge in treatment of nosocomial infections worldwide (22). There are previous studies about β -lactam resistance in Anbar province during three years ago . Shammaa and Myadaa reported that of MDR 72% , Extensively Drug XDR 24%, and PDR 4%. On other hand , a modern study documented that MDR, XDR, and PDR were 45% , 33.33% , and 22.22 % respectively (23). Ahmed reported that (42.8%) MDR , (27.5%) XDR , and (8.2%) (PDR) in Erbil province (24). The results of a study conducted by Manjula, CM (25) supported this finding. They showed that out of 41 isolates, 37 (90.2%) of them were MDR. Another study conducted by (26), Among 88 K. pneumoniae isolates, 43 (48.8%) and 20 (22.7%) were MDR and XDR, respectively.

Detection of genes using conventional PCR :

Detection of extended spectrum beta-lactamases-producing isolates genes:

Resistance to cephalosporins antibiotics is considered as an international public health threat. Throughout the world there are increasing cases of clinical infections with ESBLs, including Enterobacteriaceae such as Klebsiella pneumoniae, and E. coli. Based on Kirby-Bauer disk diffusion , confirmation test for ESBLs and ESBLs assay , 30 of the 100 ESBLs isolates were detected in this study by conventional PCR .It is worth noting, that the annealing temperature of all primers was used different , including 55 ,56 ,57 , 58 , 59 , and 60 °C .

Detection of CTX-M gene

CTX-M gene was identified by traditional PCR technique for all the present studied isolates by using specific primer that targets the specific sequence of the target gene. After that, the amplified products were carried on 1.5% agarose gel for 1.5 hr. When the agarose gel was checked under ultraviolet, one line of bands was observed in the wells of gel and at the same level for the bacterial isolates that possessed this gene. This indicates that the primer binds with its complementary sequence in the DNA strand. The results revealed that 30/30 (100%) of isolates were have this gene, whereas the bands appeared within the expected size the gene (544 bp) for all positive isolates. Figure 4.

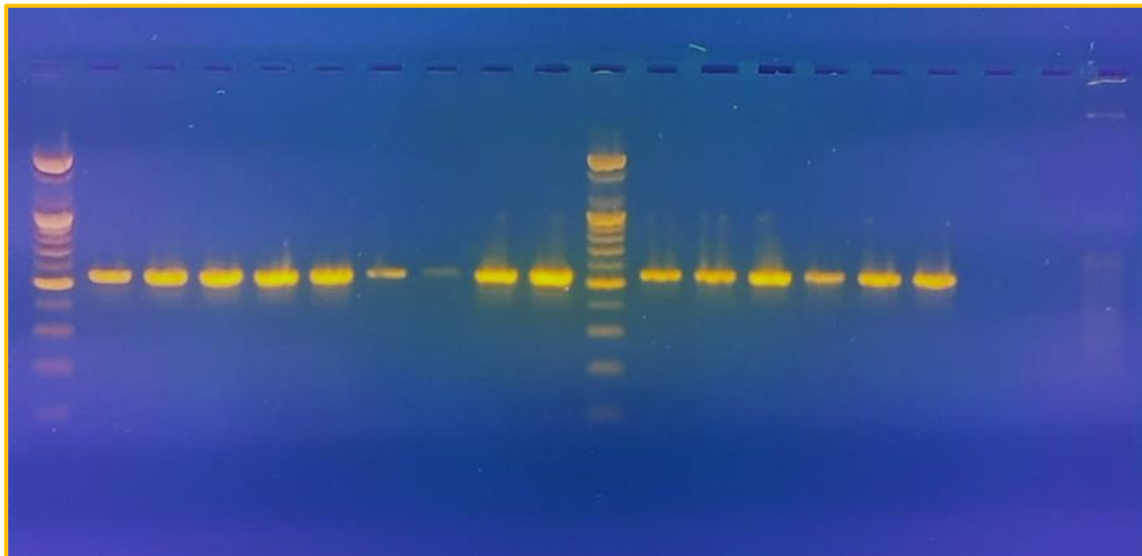


Figure 4: Conventional PCR amplification fragments for the detection of bla CTX-M gene (544) bp. Lanes 1 – 15: *Klebsiella pneumoniae*; Lane M: 100-bp DNA ladder; NC: negative control. Amplicons were electrophoresed on agarose gel (1.5%) at 70 V/cm for 1.5 hr, stained with ethidium bromid, and visualized using an UV transilluminator documentation system.

Detection of blaSHV gene :

blaSHV gene was detected by conventional PCR technique for all clinical isolates by using a specific primer. After that, the amplified products were carried on 1.1 % agarose gel for 1 hr . When the agarose gel was checked under ultraviolet, one line of bands was observed in the wells of gel and at the same level for the bacterial isolates that possessed this gene.

The results revealed that 30/30 (100%) of isolates had this gene, whereas the bands appeared within the expected size of the gene (1016 bp) for all positive isolates as shown in figure 5.

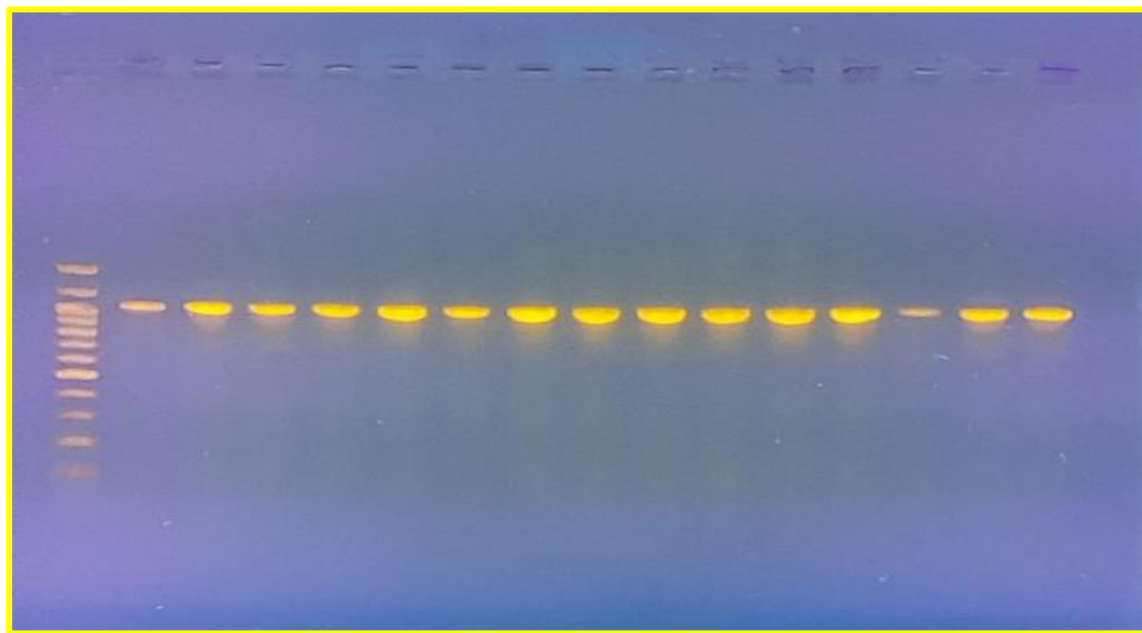


Figure 5 : Conventional PCR amplification fragments for the detection of bla SHV gene (1016) bp. Lanes 1 – 15: *Klebsiella pneumoniae*; Lane M: 100-bp DNA ladder; NC: negative control. Amplicons were electrophoresed on agarose gel (1.1%) at 70 V/cm for 1.5 hr, stained with ethidium bromid, and visualized using an UV transilluminator documentation system.

Detection of blaTEM gene :

BlaTEM gene was detected by conventional PCR technique for all clinical isolates by using specific primer. After that, the amplified products were carried on 1 % agarose gel for 1 hr . When the agarose gel was checked under ultraviolet, one line of bands was observed in the wells of gel and at the same level for the bacterial isolates that possessed this gene.

The results revealed that 30/30 (100%) of isolates were have this gene, whereas the bands appeared within the expected size the gene (1080 bp) for all positive isolates.As shown figure 6.

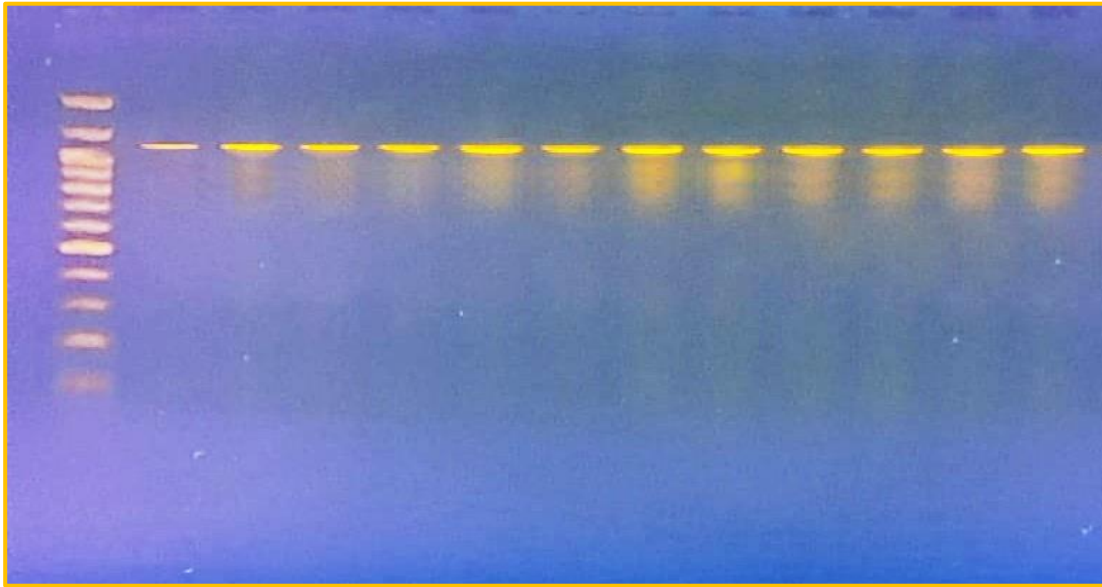


Figure 6 : Conventional PCR amplification fragments for the detection of bla TEM gene (1080) bp. Lanes 1 – 12: *Klebsiella pneumoniae*; Lane M: 100-bp DNA ladder; NC: negative control. Amplicons were electrophoresed on agarose gel (1%) at 70 V/cm for 1.5 hr, stained with ethidium bromid, and visualized using an UV transilluminator system.

As a global challenge, antimicrobial resistance in pathogenic bacteria is accompanied by high rates of mortality and morbidity. In addition, because of multidrug resistant patterns, infections have been reported to be difficult or even impossible to treat with conventional antimicrobials. Because many healthcare centers fail to diagnose causative microorganisms and their patterns of antimicrobial susceptibility timely in patients with bacteremia and other serious infections, antibiotics are broadly, liberally and mostly unnecessarily used (27).

Our results showed that occurrence of blaCTX-M gene, blaTEM gene and bla SHV gene in *K. pneumoniae* was 100%. In 2018, 22.0% *K. pneumoniae* isolated from Ramadi hospitals were resistant for carbapenem . Genotypic testing on six CRE isolates revealed that the blaOXA-48 and blaVIM genes were equally detected in these isolates, followed by blaKPC (23) ,(28). Moreover, Mohammed and Safaa reported blaFOX11 (42.3%), blaACC8 (30.7%), blaDHA 8 (30.7%), blaCIT 9(34.6%) and blaMOX 7 (26.92%) (29). Many researchers reported spread *A. baumannii* resistant to different antibiotics in Iraq hospitals . *A. baumannii* possess high capacity to acquire new resistance genes. (30),(31)

For the first time, this work identifies NDM-1 producing *Escherichia coli* isolates from Baghdad governance(32). The increasing prevalence rate of bla IMP in carbapenem –resistant *A. bauamani* and *Pseudomonas aeruginosa* isolates from wounds /Iraq(32),(33)

In this study , disseminating new genes in the province of Anbar as a result of terrorist foreign organizations of different nationalities which destroyed our town in 2014, as carbapenemases were not registered previously. Moving people to local and global cities, medical tourism and cross-border transfer of patients who are particularly involved in the development and distribution of different variants of carbapenemase encoding genes can play an important role in the development (23),(34). Figure 7 .

Awaad and laith , 2020 reported OXA-51, and OXA-23 in *A. bauammanii* in Ramadi hospitals (35),(36) . Another study by Yoser and Ali , 2021 reported blaFOX gene was detected in 13 isolates (56.5%), blaACC gene was detected in 12 isolates (52.1%) , bla DAH gen was detected in 12 isolate (52.1%),8 isolate (34.7%) had bla CIT gene , bla EBC Where was identified in 6 isolates (26.08), while the lowest percentage for bla MOX gene was identified in 5 isolates (21.7%) (37),(38) . In 2021 , Shaimaa and Mayada reported bla CTX-M2 and bla OXA1 genes (39),(40).

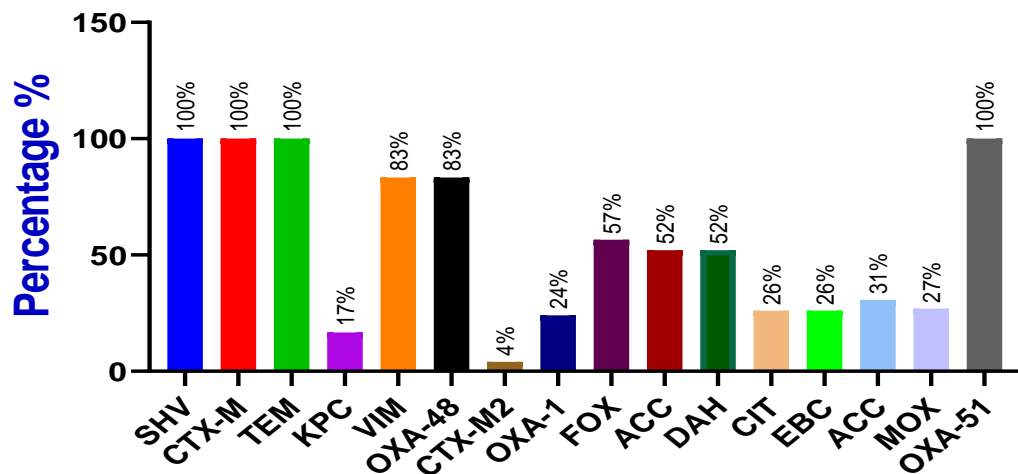


Figure 7 : Distribution of resistance genes among bacteria in Al-Ramadi hospitals from 2010 to 2022

The spread of plasmid-encoded extended-spectrum β -lactamase (ESBLs) genes (41), conferring resistance to third-generation cephalosporins including cefotaxim and ceftriaxone (42) is considered a major contributor to the ongoing emergence of antimicrobial resistance. The proportion of *Klebsiella pneumoniae* isolates displaying an ESBL-phenotype among those recovered from clinical different sources in Ramadi found here was 49%, which is in the range of values found in other countries like Kenya 12% (43) and Libya 13.4% (44). Interestingly, very different values were found in isolates also retrieved in Iran (45)(25.9%), China (41)(5.6%) or USA (46)(7%). In developing countries, patients often receive antibiotics treatment without antibiotic susceptibility testing or prescription which will exert a selective pressure on the existing *Klebsiella pneumoniae*, whereas in developed countries strategies for reducing antimicrobial use have been put in place (45). However, this result is in agreement with previous reports by Vatopoulos et al. (47)that found that *Klebsiella pneumoniae* bearing transferable Resistance plasmids were more often associated with antibiotic resistance among females than males. ESBLs positive strains from our study were highly resistant to cephalosporins, which is similar to the reports of Miao et al. (48) and Valenza et al. (49). All phenotypic ESBLs producing strains were susceptible to imipenem in agreement with reports from Tanzania(50) , Libya (51), China (52), and in contrast with the study of Hamprecht et al. (53) in Germany. The full susceptibility of isolates to imipenem may be attributable to the relatively low or non-usage of carbapenem drugs among the population. All the ESBLs positive isolates (genotypic) were resistant to tetracycline, similar to the report from Egypt (54) and Libya (51) (88.9% resistance), which could be due to an extensive use of this drug among humans and animals.

blaCTX-M was very prevalent among the ESBL-positive isolates, in agreement with previous studies suggesting this gene is widespread worldwide (48) , (54) , (51), although blaTEM-1 was also found in approximately three quarter of all ESBL isolates tested. No data on the presence of ESBL associated genes was available for clinical isolates in Nigeria. The range of values reported from Egypt (55) (73.7% blaCTX-M), Turkey (56) (73.43% blaTEM) and USA (46) (74% blaCTX-M). This study also shows that the carriage of multiple bla genes complicates the phenotypic interpretation of the resistance phenotypes, which is related to complex antimicrobial resistance (57). Isolates with multiple combinations of bla genes and especially those carrying blaCTX-M+blaTEM and blaCTX-M+blaTEM+blaSHV were resistant to a larger number of β -lactams (>92% resistance). blaCTX-M in combination with blaTEM gene was found in 46.7% of the isolates, similar to the findings of Harada et al.(58), which reported 48.8% of *E. coli* carried blaCTX-M+blaTEM in clinical isolates from Japanese tertiary hospital, but higher than the result of Tawfick et al. (54) who found only 21.7% of *E. coli* isolates from diarrhoeic stool in Egypt carrying both blaCTX-M and blaTEM.

Moreover, the development of resistance to cephalosporins may be due to intrinsic or acquired resistance mechanisms or both. Large numbers of bacteria, both commensals and pathogens, naturally tend to be resistant to certain classes of antimicrobial agents. This insensitivity is termed intrinsic resistance, the occurrence of which limits and complicates drug selections for treatment. This can increase the risk of the development of acquired resistance. Gram-negative organisms reduce the uptake of β -lactam drugs, by selectively altering their cell membrane porin channels. The reduction of outer membrane permeability in this manner prevents the β -lactams from reaching their targets.

Conclusion:

It can be concluded that antimicrobial resistance in *K. pneumoniae* is a clear and present danger in Anbar province which needs strong surveillance to curb this menace. Although low resistance rate to carbapenem, Amikacin, Ciprofloxacin, Levofloxacin, Tigecycline were recorded in this thesis, more cautious efforts should be made to develop a new line of antimicrobials as resistance to these drugs are surging. The present study revealed a high level of *K. pneumoniae* strains harboring extended spectrum β -lactamases in our hospitals, which may lead to the dissemination of multiple antibiotic resistance.

REFERENCES

1. Effah CY, Sun T, Liu S, Wu Y. Klebsiella pneumoniae: An increasing threat to public health. *Ann Clin Microbiol Antimicrob*. 2020;19(1):1–9.
2. Bhatia P, Sharma A, George AJ, Anvitha D, Kumar P, Dwivedi VP, et al. Antibacterial activity of medicinal plants against ESKAPE: An update. *Heliyon*. 2021;7(2):e06310.
3. Pillai D, Kalasseril SG. Genetic diversity and prevalence of extended spectrum beta-lactamase producing *Escherichia coli* and *Klebsiella pneumoniae* in aquatic environment receiving untreated hospital effluents. 2022;
4. Bergšpica I, Kaprou G, Alexa EA, Prieto M, Alvarez-Ordóñez A. Extended spectrum β -lactamase (ESBL) producing *Escherichia coli* in pigs and pork meat in the European Union. *Antibiotics*. 2020;9(10):678.
5. Miftode I-L, Pasare M-A, Miftode R-S, Nastase E, Plesca CE, Lunca C, et al. What Doesn't Kill Them Makes Them Stronger: The Impact of the Resistance Patterns of Urinary Enterobacteriales Isolates in Patients from a Tertiary Hospital in Eastern Europe. *Antibiotics*. 2022;11(5):548.
6. Elbehiry A, Aldubaib M, Al Rugaie O, Marzouk E, Abaalkhail M, Moussa I, et al. Proteomics-based screening and antibiotic resistance assessment of clinical and sub-clinical *Brucella* species: An evolution of brucellosis infection control. *PLoS One*. 2022;17(1):e0262551.
7. Joseph L, Merciecca T, Forestier C, Balestrino D, Miquel S. From *Klebsiella pneumoniae* Colonization to Dissemination: An Overview of Studies Implementing Murine Models. *Microorganisms*. 2021;9(6):1282.
8. Rodríguez-Beltrán J, DelaFuente J, Leon-Sampedro R, MacLean RC, San Millán A. Beyond horizontal gene transfer: the role of plasmids in bacterial evolution. *Nat Rev Microbiol*. 2021;19(6):347–59.
9. Bauer AW, Kirby WMM, Sherris JC, Turck M. Antibiotic susceptibility testing by a standardized single disk method. *Am J Clin Pathol*. 1966;45(4-ts):493–6.
10. Vandepitte J, Verhaegen J, Engbaek K, Rohner P, Piot P, Heuck CC, et al. Basic laboratory procedures in clinical bacteriology. *World Health Organization*; 2003.
11. Kadum SM. Colistin Susceptibility in Carbapenem Resistant *Klebsiella pneumoniae* and their Ability of Biofilm Formation. *Iraqi J Sci*. 2020;517–27.
12. Namratha KG, Sreeshma P, Subbannayya K, Dinesh P V, Champa H. Characterization and antibiogram of *Klebsiella* spp. isolated from clinical specimen in a rural teaching hospital. *Sch J Appl Med Sci*. 2015;3(2E):878–83.
13. Nirwati H, Sinanjung K, Fahrulnissa F, Wijaya F, Napitupulu S, Hati VP, et al. Biofilm formation and antibiotic resistance of *Klebsiella pneumoniae* isolated from clinical samples in a tertiary care hospital, Klaten, Indonesia. In: *BMC proceedings*. BioMed Central; 2019. p. 1–8.
14. Hasan SA, Abass KS. Prevalence of Gram Negative Bacteria Isolated from Patients with Burn Infection and their Antimicrobial Susceptibility Patterns in Kirkuk City, Iraq. *Indian J Public Heal Res Dev*. 2019;10(8).
15. Hasan SA, Najati AM, Abass KS. Prevalence and antibiotic resistance of “*pseudomonas aeruginosa*” isolated from clinical samples in Kirkuk City, Iraq. *Eurasia J Biosci*. 2020;14(1):1821–5.
16. Alves MS, Dias RC da S, de Castro ACD, Riley LW, Moreira BM. Identification of clinical isolates of indole-positive and indole-negative *Klebsiella* spp. *J Clin Microbiol*. 2006;44(10):3640–6.
17. Finegold SM, Martin WJ. Bailey and Scotts diagnostic microbiology. In: *Bailey and Scotts diagnostic microbiology*. 1982. p. 705.
18. Funke G, Monnet D, deBernardis C, von Graevenitz A, Freney J. Evaluation of the VITEK 2 system for rapid identification of medically relevant gram-negative rods. *J Clin Microbiol*. 1998;36(7):1948–52.
19. Barenfanger J, Drake C, Kacich G. Clinical and financial benefits of rapid bacterial identification and antimicrobial susceptibility testing. *J Clin Microbiol*. 1999;37(5):1415–8.
20. Doern G V, Vautour R, Gaudet M, Levy B. Clinical impact of rapid in vitro susceptibility testing and bacterial identification. *J Clin Microbiol*. 1994;32(7):1757–62.
21. Clinical And Laboratory Standards Institute. M100 Performance Standards for Antimicrobial Susceptibility Testing. Vol. 8, *Journal of Services Marketing*. 2018. 31 p.
22. Cao X, Xu X, Zhang Z, Shen H, Chen J, Zhang K. Molecular characterization of clinical multidrug-resistant *Klebsiella pneumoniae* isolates. *Ann Clin Microbiol Antimicrob*. 2014;13(1):1–5.
23. Ahmed MM, Al Meani SAL. Occurrence of *Klebsiella pneumoniae* carbapenemase KPC gene in *Klebsiella pneumoniae* isolated from patients in Anbar city of Iraq. *Ann Trop Med Public Heal*. 2019;22(Special Issue 5).
24. Ahmed HJ, Ganjo AR. Detection of carbapenemase-producing *Klebsiella pneumoniae* and *Escherichia coli* recovered from clinical specimens in Erbil City Kurdistan Region of Iraq. *Al-Mustansiriyah J Sci*. 2019;30(2):10–8.

25. Manjula NG, Math GC, Nagshetty K, Patil SA, Gaddad SM, Shivannavar CT. Antibiotic susceptibility pattern of ESBL producing *Klebsiella pneumoniae* isolated from urine samples of pregnant women in Karnataka. *J Clin diagnostic Res JCDR*. 2014;8(10):DC08.
26. Moghadam MT, Shariati A, Mirkalantari S, Karmostaji A. The complex genetic region conferring transferable antibiotic resistance in multidrug-resistant and extremely drug-resistant *Klebsiella pneumoniae* clinical isolates. *New microbes new Infect*. 2020;36:100693.
27. Akova M. Epidemiology of antimicrobial resistance in bloodstream infections. *Virulence*. 2016;7(3):252–66.
28. Jain MS, Barhate SD. Tigecycline is the First Clinically-Available Drug in a new class of Antibiotics called the Glycylcyclines: A Review. *Asian J Pharm Technol*. 2020;10(1):48–50.
29. Ibrahim MO, Al Meani SAL. DETECTION OF PLASMID-MEDIATED AMPC β -LACTAMASE GENES AND EVALUATION THE SYNERGISTIC EFFECT OF CLOVE VOLATILE OIL AND ANTIBIOTICS IN CLINICAL ISOLATES OF *KLEBSIELLA PNEUMONIA* IN IRAQ.
30. Eveillard M, Kempf M, Belmonte O, Pailhoriès H, Joly-Guillou M-L. Reservoirs of *Acinetobacter baumannii* outside the hospital and potential involvement in emerging human community-acquired infections. *Int J Infect Dis*. 2013;17(10):e802–5.
31. Abozed MF, Abd El Latif HK, Serry FM, El Sayed LM. Activity of commonly used intravenous nutrient and bisolvon in neonatal intensive care units against biofilm cells and their synergetic effect with antibiotics. *Asian J Pharm Technol*. 2013;3(2):81–90.
32. Singh H, Kaur R, Singh S. Use of *Syzygium aromaticum* (Clove) in combination with standard antibiotics: Application in antimicrobial chemotherapy. *Res J Pharm Technol*. 2018;11(5):1815–9.
33. Thyagarajaan R, Namasivayam SKR, Narendrakumar G, Singh V, Samyudurai S. Evaluation of in Vitro Drug Controlled Release of Biocompatible Metallic and Non Metallic Nanoparticles Incorporated Anti Bacterial Antibiotics and Their Anti Biofilm Activity Against *E. coli*. *Res J Pharm Technol*. 2015;8(3):316.
34. Rupashri S V, Karpagavalli S. Benefits of Prophylactic Antibiotics in Third Molar Impaction. *Res J Pharm Technol*. 2015;8(8):1028–32.
35. HameAed AM, Najeeb LM. Molecular Detection of blaOXA-51 in Carbapenem-resistant *Acinetobacterbaumannii* Isolated from Different Clinical Sources. *Med Leg Updat*. 2020;20(4):693–6.
36. Gayathri A, Madhanraj P, Panneerselvam A. Diversity, Antibacterial Activity And Molecular Characterization of Actinomycetes Isolated From Salt Pan Region of Kodiakarai, Nagapattinam DT. *Asian J Pharm Technol*. 2011;1(3):79–81.
37. Ragheb YM, Abdulkarim AH. Phenotypic and Genotypic Detection Ampc β -Lactamase Producing *E. coli* Isolated from UTI in Anbar Governate. *Ann Rom Soc Cell Biol*. 2021;1181–92.
38. Hemalatha K, Raj DN, Begam MF, Sharanya VK, Girija K. Synthesis, Characterization, Docking study and Anti-Bacterial Evaluation of Benzimidazole Derivatives as Biotin Carboxylase Inhibitors. *Asian J Pharm Technol*. 2017;7(2):109–14.
39. Ahmed SH, Shehan MA. Phenotypic and Genotypic Detection of Extended Spectrum β -lactamase in MDR-*Klebsiella pneumoniae* in Anbar Governate. *Ann Rom Soc Cell Biol*. 2021;4984–92.
40. Kale DR, Khedkar AN, Pawar JB. Formulation and in-vitro Evaluation of floating tablets of Antibiotics used in the treatment of Peptic ulcer. *Asian J Pharm Technol*. 2021;11(1).
41. Bai L, Wang L, Yang X, Wang J, Gan X, Wang W, et al. Prevalence and molecular characteristics of extended-spectrum β -lactamase genes in *Escherichia coli* isolated from diarrheic patients in China. *Front Microbiol*. 2017;8:144.
42. DD Pitout J. Enterobacteriaceae that produce extended-spectrum β -lactamases and AmpC β -lactamases in the community: the tip of the iceberg? *Curr Pharm Des*. 2013;19(2):257–63.
43. Too JR, Ngari M, Kikui GM, Matey EJ, Koima J, Chebii J, et al. Prevalence, virulence genes and Antimicrobial Resistance of Shiga-toxigenic *E. coli* in diarrhoea patients from Kitale, Kenya. *Afr J Health Sci*. 2017;30(2):105–19.
44. Bassyouni RH, Gaber SN, Wegdan AA. Fecal carriage of extended-spectrum β -lactamase-and AmpC-producing *Escherichia coli* among healthcare workers. *J Infect Dev Ctries*. 2015;9(03):304–8.
45. Alizade H, Fallah F, Ghanbarpour R, Aflatoonian MR, Goudarzi H, Sharifi H. Phylogenetic groups, extended-spectrum β -lactamases and metallo- β -lactamase in *Escherichia coli* isolated from fecal samples of patients with diarrhea in Iran. *Gastroenterol Hepatol From Bed to Bench*. 2015;8(3):207.
46. Chandramohan L, Revell PA. Prevalence and molecular characterization of extended-spectrum- β -lactamase-producing Enterobacteriaceae in a pediatric patient population. *Antimicrob Agents Chemother*. 2012;56(9):4765–70.
47. Vatopoulos AC, Varvaresou E, Petridou E, Moustaki M, Kyriakopoulos M, Kapogiannis D, et al. High rates of antibiotic resistance among normal fecal flora *Escherichia coli* isolates in children from Greece. *Clin Microbiol Infect*. 1998;4(10):563–9.
48. Miao Z, Li S, Wang L, Song W, Zhou Y. Antimicrobial resistance and molecular epidemiology of ESBL-producing *Escherichia coli* isolated from outpatients in town hospitals of Shandong Province, China. *Front Microbiol*. 2017;8:63.
49. Valenza G, Nickel S, Pfeifer Y, Eller C, Krupa E, Lehner-Reindl V, et al. Extended-spectrum- β -lactamase-producing *Escherichia coli* as intestinal colonizers in the German community. *Antimicrob Agents Chemother*. 2014;58(2):1228–30.
50. Tellevik MG, Blomberg B, Kommedal Ø, Maselle SY, Langeland N, Moyo SJ. High prevalence of faecal carriage of ESBL-producing Enterobacteriaceae among children in Dar es Salaam, Tanzania. *PLoS One*. 2016;11(12):e0168024.
51. Ahmed SF, Ali MMM, Mohamed ZK, Moussa TA, Klena JD. Fecal carriage of extended-spectrum β -lactamases and AmpC-producing *Escherichia coli* in a Libyan community. *Ann Clin Microbiol Antimicrob*. 2014;13(1):1–8.
52. Xu G, An W, Wang H, Zhang X. Prevalence and characteristics of extended-spectrum β -lactamase genes in *Escherichia coli* isolated from piglets with post-weaning diarrhea in Heilongjiang province, China. *Front Microbiol*. 2015;6:1103.
53. Rohde AM, Zweigner J, Wiese-Posselt M, Schwab F, Behnke M, Kola A, et al. Prevalence of third-generation cephalosporin-resistant Enterobacteriales colonization on hospital admission and ESBL genotype-specific risk factors: a cross-sectional study in six German university hospitals. *J Antimicrob Chemother*. 2020;75(6):1631–8.
54. Sinha R, Kamath S, M Shenoy S. Association of risk factors, antimicrobial resistance trends and occurrence of blaTEM, blaSHV and blaCTX M in *Escherichia coli* causing bacteremia. *Infect Disord Targets (Formerly Curr Drug Targets-Infectious Disord)*. 2016;16(2):95–100.
55. Tawfick MM, El-Moghazy AA, Hassan MA. PCR-Based Molecular Detection of ESBLs Encoding Genes blaTEM, blaCTX-M and blaSHV among MDR *Escherichia coli* Isolates from Diarrhoea Stool Cultures in Cairo, Egypt. *Int J Res Stud Microbiol Biotechnol*. 2016;2:7–14.
56. Bali EB, Accedil L, Sultan N. Phenotypic and molecular characterization of SHV, TEM, CTX-M and extended-spectrum-lactamase produced by *Escherichia coli*, *Acinetobacter baumannii* and *Klebsiella* isolates in a Turkish hospital. *African J Microbiol Res*. 2010;4(8):650–4.
57. Polse RF, Yousif SY, ASSAFI MS. Prevalence and molecular characterization of extended spectrum beta-Lactamases-producing uropathogenic *Escherichia coli* isolated in Zakho, Iraq. *J Microbiol Infect Dis*. 2016;6(4):163–7.
58. Harada Y, Morinaga Y, Yamada K, Migiyama Y, Nagaoka K, Uno N. Clinical and molecular epidemiology of extended-spectrum β -lactamase-producing *Klebsiella pneumoniae* and *Escherichia coli* in a Japanese tertiary hospital. *J Med Microb Diagn*. 2013;2(127):2161–703.