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Study the Frequency of ESBL Genes in Klebsiella Pneumoniae Isolated from Clinical Samples

Hassan Khaled Ali, Ammar Hassan Ali

Abstract: **Introduction and purpose:** The acquisition of broad-spectrum beta-lactamase producing enzymes has led to an increase in the resistance of Klebsiella pneumoniae strains to beta-lactam antibiotics. Usually, the genes encoding beta-lactamases are located on the plasmid and can be transferred to other gram-negative strains. The aim of the present study was to determine the pattern of resistance to β-lactam antibiotics as well as the frequency of broad-spectrum beta-lactamase genes in Klebsiella pneumoniae isolates obtained from clinical samples.

Methods: In this descriptive-cross-sectional study, 750 clinical samples were examined for the isolation of Klebsiella pneumoniae bacteria by culture method and biochemical tests. The antibiotic resistance pattern of the isolates was determined by disk diffusion method to ampicillin, amoxicillin, meropenem, cefixime, ciprofloxacin and cefotaxime antibiotics. Also, in order to isolate beta-lactamase-producing isolates, the combined cefotaxime-clavulanic acid disc method was used. The frequency of resistance genes of bla SHV, bla TEM and bla CTX genes was evaluated by PCR method using specific primers.

Results: 62 isolates were identified as Klebsiella pneumoniae. The rate of resistance to ampicillin, amoxicillin, imipenem, cefixime, ciprofloxacin and cefotaxime antibiotics was: 94, 95, 24, 59, 47 and 52%, respectively. 37 of the isolates were beta-lactamase producers. Also, 54 isolates (87%) had the bla CTX-M gene, 37 (59%) had the bla SHV gene, and (98.4%) 61 isolates had the bla TEM gene.

Conclusion: This study showed that the spread of Klebsiella pneumoniae isolates producing broad-spectrum beta-lactamases has increased in clinical samples. Therefore, continuous monitoring of broad-spectrum beta-lactamase-producing bacteria and determination of their resistance patterns can help reduce the spread of these resistant species in society.

Key points: Klebsiella pneumoniae, antibiotic resistance, broad spectrum beta-lactamases. PCR.

Introduction

Klebsiella is a gram-negative bacillus belonging to the Enterobacteriaceae family, which has a relatively close genetic relationship with other genera of this family, such as Escherichia, Salmonella, Shigella, and Yersinia. Members of the Klebsiella genus are non-motile bacteria that ferment lactose and sucrose and decarboxylate lysine, but do not decarboxylate ornithine and slowly hydrolyze urea. Klebsiella pneumoniae was formerly called Friedlander's bacillus because they have large, mucoid and moist colonies due to the presence of a capsule. Klebsiella oxytoca is the only indole-positive Klebsiella, able to grow at 10°C and capable of using benzoate, and along with Klebsiella pneumoniae, it is methyl red negative. Klebsiella produces a very slimy pink colony on the surface. These bacteria are usually encapsulated. Most strains produce gas from sugars. Klebsiella pneumoniae is the indicator bacterium of this group of bacteria (1).

Klebsiella species are ubiquitous in nature. Klebsiella probably has two common habitats, one of which is the environment, these bacteria are found in surface water, sewage, soil, and on plants, and the second habitat is the mucosal surfaces of mammals, including humans, horses, or pigs. In this

sense, the genus Klebsiella is similar to Enterobacter and Citrobacter, but it is different from Shigella and Escherichia coli, which are common in humans but not common in the environment. In humans, Klebsiella pneumoniae exists as a saprophyte in the nasopharynx and intestinal tract. Its isolation rate in stool samples is between 5-38% and from nasopharynx between 1-6%. Because Gram-negative bacteria do not find good conditions for growth on human skin, Klebsiella species are rarely found in these areas and are considered as temporary members of the skin flora. This level of transmission is different in the hospital environment; For example, the intensity of colonization increases with the length of hospitalization. Apart from medical equipment (contamination due to faulty hygiene practices) and blood products, the main reservoirs of Klebsiella transmission in the hospital are the digestive system of patients and the hands of hospital personnel. The ability of this organism to spread rapidly often leads to nosocomial infections, especially in the neonatal unit. According to the Centers for Disease Control and Prevention in the United States of America, Klebsiella species account for about 8% of endemic hospital infections and 3% of epidemic outbreaks. Epidemic nosocomial infections caused by multi-resistant strains are very dangerous and scary. Reports indicate that about one third of people are carriers of this bacterium. The rate of establishment of these bacteria in hospitalized patients is higher than that of outpatients. These bacteria are one of the important factors of community and hospital acquired infections and also cause a wide range of infections including septicemia, pneumonia, urinary tract infection, meningitis and purulent abscesses in different organs, especially liver abscesses (2).

Material and methods

Cultivation environments, materials and devices used

Table 1: Consumables used in this research

Producing country	Manufacturer	name of the material	Row	Producing country	Manufacturer	name of the material	Row
Antibody medicine	Iran	Disc oxidase	11	Germany	Merc	Nutrients if	1
Antibody medicine	Iran	Warm coloring kit	12	Germany	Merc	Broth nutrient	2
Germany	Merc	OF environment	13	Germany	Merc	TSI	3
Irwin Chemistry	Iran	Glycerol	14	Germany	Merc	Urea environment	4
Germany	Merc	SIM	15	Germany	Merc	Mullerhinton Agar	5
Irwin Chemistry	Iran	ethanol	16	Germany	Merc	Urea Broth	6
Irwin Chemistry	Iran	Peroxide	17	Germany	Merc	Blood if	7
Irwin Chemistry	Iran	Antibiotic disc	18	Germany	Merc	Simon Citrate	8
Irwin Chemistry	Iran	EMB agar	19	Germany	Merc	MR-VP	9
	Iran	Primer	20	Germany	Merc	Mueller Hinton Agar	10
Germany	Merc	TSB	21	Iran	Karmania Pars Gen	Extraction kit	11

Table 2: Devices needed in this research

Manufacturing Country	Device model	device name	Row	Manufacturing Country	Device model	device name	Row
Iran	The leader of medicine	autoclave	6	Germany	Sartoris	digital Balance	1
Iran	Behdad	Ben Murray	7	Iran	Behdad	Incubator	2
Iran	Pars Azma	Laminar flow	8	Iran	Behdad	Stylish incubator	3
Iran	The leader of medicine	autoclave	9	Iran	T180 Plasma 103	Spectrophotometer	4
Iran	gentra	heater	10	Germany	Zeiss	optical microscope	5
Iran	U-320-R BOECO	centrifuge		Iran	Behdad	Fixed incubator	6
		Gel Dock		Iran	Behdad	shaker	7
						PCR (thermal cycler)	

Sample collection

The current research is a descriptive, cross-sectional (experimental) research. The urine samples of people suspected of having a urinary tract infection The number of tested samples was 750 samples, and after proper training, the samples were taken from the middle urine of people in sterile plastic containers with lids.

Culture environments

Streak (linear) culture was performed from each urine sample under a laminar hood under sterile conditions on eosin methylene blue agar and blood agar. The calibrated loop whose ring diameter is 4 mm was removed (the volume removed from the liquid sample when the ring diameter is 4 mm will be equal to 0.01 cc).

After cultivation, the samples were placed at 37 oC for 24 hours and after the end of the incubation period, they were evaluated in terms of growth and growth rate. Since the number of bacteria in each milliliter of urine sample, which is considered as an indicator of urinary infection, is of particular importance, in this way, by counting the colonies and multiplying by 100 (image of the removed volume), the number of bacteria in each A milliliter of sample was calculated, the number of bacteria less than 1000 was considered as negative and the number of bacteria equal to or more than 100000/ml was considered as positive as a sign of urinary infection (3).

TSI environment

TSI biochemical medium contains three sugars: glucose 0.1%, sucrose 1% and lactose 1%. This medium contains sodium thiosulfate, iron salt, phenol red (pH reagent) and peptone (source of carbon and nitrogen). If only glucose is fermented, due to low acid production, only the end surface of the culture tube will turn yellow. Also, if lactose and sucrose are fermented, due to high acid production, both the surface and the depth of the tube will turn yellow. The main reagent in this environment is phenol red, which will be yellow in acidic pH and red in alkaline pH. Klebsiella pneumoniae strains turn the color of this environment yellow due to the fermentation of sugars, but they are not able to produce hydrogen sulfide. Also, gas production in this environment will be positive for most strains. (4).

Cultivation on Simon Citrate Agar medium

Bacteria that have the ability to use citrate as the only source of carbon and energy, as soon as citrate enters the cell cytoplasm, the bacterial enzyme converts citrate into pyruvate and CO2. It

combines and forms the alkaline composition of sodium carbonate. Bromothymol blue pH reagent changes color to blue in alkaline pH. The color change of the culture medium from green to blue is a positive sign of the test. In sterile conditions, a small amount of The bacterial colony was inoculated in a Simon Citrate Agar gradient tube by sterile anus and placed in an incubator at 35 oC for 72 hours, and the tubes were checked every day for color change and bacterial growth. Klebsiella pneumoniae strains, due to the presence of citratease enzyme, convert citrate into one of the alkaline compounds (5 , 6).

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Antibiogram test by disc diffusion method

Determining the sensitivity of Klebsiella pneumoniae bacteria to several beta-lactam antibiotics was done by standard Kirby & Bauer method. First, a standard suspension with a concentration of 1.5 x 108/ml equivalent to half McFarland of each bacterium was prepared in physiological serum, and then the antibiogram was performed by disc diffusion method on Mueller Hinton agar culture medium.

Antibiogram

Mueller Hinton Agar medium was used to perform antibiogram. Ampicillin, amoxicillin, imipenem, cefixime, ciprofloxacin and cefotaxime discs were used in this study. To prepare the microbial suspension in this experiment, 3-4 colonies of freshly purified bacteria were removed with the help of a swab and placed in a tube containing physiological serum in the form of a suspension until the turbidity equal to the turbidity of the standard tube (0.5 McFarland solution) was obtained. After immersing the sterile swab in the bacterial suspension, take the excess solution by pressing the swab to the side of the tube and then spread it on the surface of the plate at an angle of 60 degrees to each other and finally rotate the swab around the inner part of the plate and then put it inside the disinfectant given. Antibiotic disks, which were previously taken out of the refrigerator and their temperature reached room temperature, were placed on the surface of the culture medium using tweezers that were placed in alcohol and then cooled with a sterile flame, and with the tip of the tweezers It is placed in place until all contact is complete. The discs were placed with a distance of 15 mm from the edge of the plate and 24 mm from the center of one disc to the center of the other disc. Place the plates at 35°C for 18-24 hours, and then measure the lack of growth in millimeters using a precise ruler, and use the CLSI 2021 table to determine the sensitivity of the bacteria to the antibiotic. It was reported as semi-sensitive and resistant (7).

Determination of ESBL resistance genes

In order to determine resistance genes, each Klebsiella pneumoniae isolate was cultured in liquid medium and after complete growth, quality DNA extraction was performed. Then, using special primers related to the identification of resistance genes, PCR test was performed according to the relevant temperature protocol. The primers were prepared from the articles and ordered to Synaclone for synthesis. The PCR product was electrophoresed with a 1% gel and the formed bands were visualized by a gel document device.

DNA extraction

First, bacteria were cultured in nutrient broth culture medium. Culture of pure bacteria was done in 10 ml of the above medium and placed in an incubator for 24 hours at a temperature of 30 degrees Celsius. DNA extraction was performed using the Karmania kit of Parsgene, Iran with the catalog CN: KPG-GNB. This kit is used to extract DNA from Gram-negative bacteria. The tubes containing the 24-hour culture of bacteria were centrifuged for 5 minutes at 4000 rpm and the supernatant was removed. The remaining sediment was poured into a 1.5 microtube and DNA extraction was performed according to the following steps:

1. The kit is taken out of the refrigerator to reach room temperature. The lysis solution was placed at 37 °C for 10 min and gently shaken.

2. 100 µL of sample was mixed with 400 µL of lysis solution and vortexed for 15-20 seconds. At this stage, a homogenized suspension should be obtained (if it is fragmented, it should be made uniform by pipetting).
3. 300 microliters of precipitation solution was added and mixed by vortexing for 5 seconds. Then the solution was transferred to a spin column with another collection tube and then centrifuged for 1 minute at 12,000 rpm.
4. 500 microliters of washing buffer number 1 was added to the sample and centrifuged for 2 minutes at 9000 rpm, and then the collected liquid was drained into the collection tube.
5. 60 microliters of sterile deionized water was added and kept for 3 minutes at a temperature of 60-65 degrees Celsius.
6. 400 microliters of washing buffer No. 2 was added to the sample and centrifuged for 1 minute at 12000 rpm, and then the collected liquid was drained into the collection tube.
7. Centrifugation was performed for 1 minute at 12,000 rpm and the DNA genetic material was separated in the bottom tube and used for the next stage of PCR.

Polymerase chain reaction (PCR)

In this step, the template DNA was first poured into the Eppendorf tubes and then the master mix was added to it. The ingredients in Master Mix are listed in the table below.

Table 3: Quantities of materials used to perform the PCR reaction

PCR mix	volume (ml)
PCR buffer	5/2
dNTP	0.5
Forward primer	1
Reverse primer	1
Taq polymerase	0.2
$Mgcl_2$ solution	0.75
DNA	2
dH_2o	17
Total	25

Table 4: Primers used in PCR

Target gene	**Product length bp**	**Primer sequence '5 to'3**	
Bla-SHV	753	ATGCGTTATATTCGCCTGTG TGCTTTGTTATTCGGGCCAA	(Ehlers et al., 2009)
Bla TEM	445	TCGCCGCATACACTATTCTCAGAATGA ACGCTCACCGGCTCCAGATTTAT	(Ehlers et al., 2009)
Bla CTX-M-	551	CGCTTTGCGATGTGCAG ACCGCGATATCGTTGGT	(Amiri et al.,2016)

Table 5: Temperature prowgram of the thermocycler device to perform PCR

period of time	Temperatures	Step name
10 minutes	94	Primary denaturation
1 minute	94	Final denaturation
1 minute	60	annealing of primers
1 minute	72	Initial extension
7 minutes	72	Final extension
30 cycles		Number of cycles

Results

The results of samples contamination with Klebsiella pneumoniae bacteria

In this study, 750 samples were collected, of which 62 samples (...%) were infected with Klebsiella pneumoniae. The isolated samples were confirmed in the microbiology laboratory by conducting microbiological and biochemical tests

Blood agar and EMB media were used to isolate clinical isolates. After 24 hours of incubation, large and strongly mucoid colonies were formed.

Figure 1: Klebsiella pneumoniae growth in blood agar (right) and mechanical (left) culture media

The results of biochemical tests to diagnose Klebsiella pneumoniae

Klebsiella pneumoniae isolates with characteristics, growth in blood agar and mechanical culture media, fermentation reaction of all three sugars in TSI culture medium, Simon Citrate was positive, movement was negative, indole was negative, hydrogen sulfide was negative, MR was negative and VP was positive.

The result of testing and investigation in different cultivation environments

The results of the maximum test were checked within 24 hours after cultivation and the results were interpreted according to the following tables

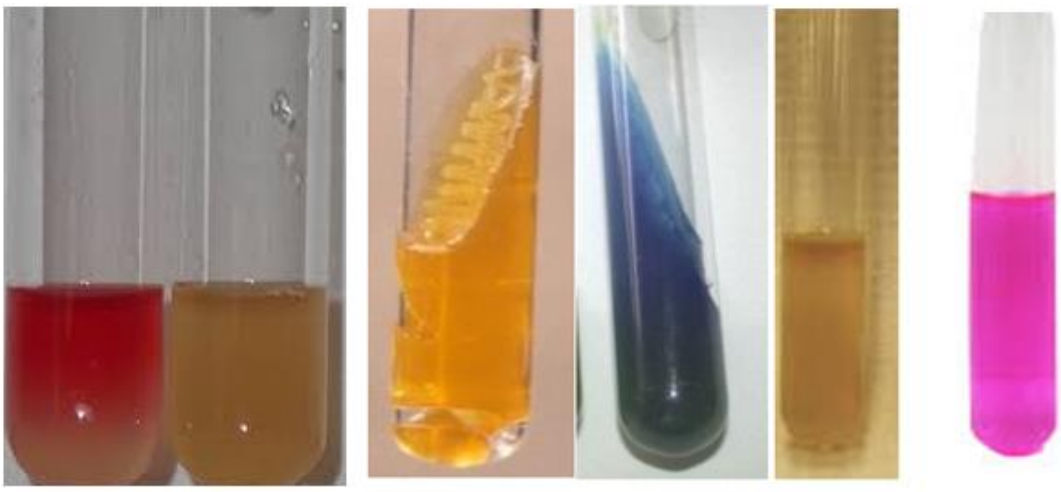

Figure 2: The results of TSI, Simon Citrate, SIM, Urea, MR (negative) and VP (positive) tests.

Results of determination of antibiotic resistance pattern by disc diffusion method

All strains of Klebsiella pneumoniae after isolation and definitive diagnosis were examined to perform the disc diffusion test. The diameter of the growth halo around the discs was measured using a millimeter ruler and evaluated according to CLSI 2021 standard tables. The samples were reported as resistant (R), semi-sensitive (I), and sensitive (S). The results of the antibiogram of 62 Klebsiella pneumoniae isolates showed that the rate of resistance to the antibiotics ampicillin: 94%,

amoxicillin: 95%, imipenem: 24%, cefixime: 59%, ciprofloxacin: 47% and cefotaxime: 52%. In all cases, a high level of antibiotic resistance is seen (Tables 4-4, 1-2, 4-3, and 4-4). Also, the combined disk results showed that 37% of Klebsiella pneumoniae isolates were beta-lactamase producers.

Table 6: Results of antibiogram by disk diffusion method of Klebsiella pneumoniae isolates number 1-20.

	Bacteria code	AM	AMX	IPM	CFM	CP	CTX	CTC
	1	20 S	20 S	17 R	R	R	16 R	22
	2	R	R	22 Sm	R	20 R	30 S	30
	3	R	R	R	R	R	R	R
	4	R	R	32 S	28 S	35 S	32 S	33
	5	22 S	25 S	18 R	R	R	20 R	37
	6	20 S	R	50 S	38 S	40 S	40 S	40
	7	R	R	30 S	10 R	26 S	15 R	28
	8	R	R	R	R	R	R	R
	9	R	R	30 S	28 S	35 S	R	18
	10	R	R	30 S	18 Sm	40 S	30 S	31
	11	R	R	R	R	R	40 S	38
	12	R	R	30 S	30 S	35 S	32 S	30
	13	R	R	31 S	R	R	R	R
	14	R	R	30 S	R	28 S	9 R	18
	15	13 R	R	31 S	25 S	35 S	34 S	30
	16	R	R	22 Sm	R	22 Sm	12 R	21
	17	R	R	R	R	R	R	R
	18	R	R	R	R	R	R	R
	19	R	R	30 S	18 Sm	28 S	35 S	36
	20	R	R	35 S	34 S	35 S	35 S	36

Table 7: General results of antibiogram of 62 Klebsiella pneumoniae isolates

(%)resistant	(%)semi sensitive	(%)sensitive	Amount of disc in micrograms	Symbol	Antibiotics
94	-	6	10µg	AM	Ampicillin
95	-	5	30 µg	AMX	Amoxicillin
24	8	68	10 µg	IMP	Amy Panam
59	3	38	5 µg	CFM	Cefixime
47	10	43	30µg	CP	Ciprofloxacin
52	-	48	30 µg	CTX	Cefotaxime

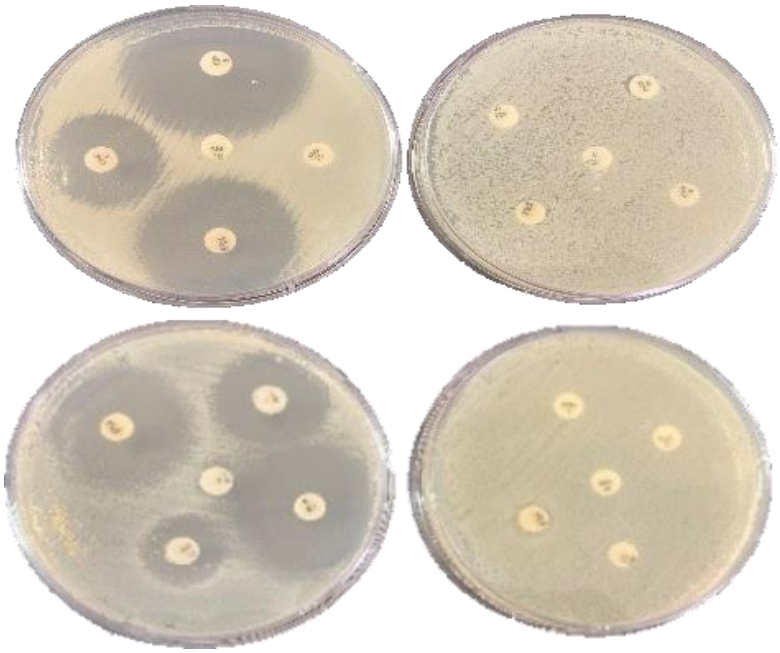

Figure 3: Example of antibiogram test results

Molecular test findings

Investigating the presence of SHV, TEM and CTX β-lactamase genes in Klebsiella pneumoniae isolates

The presence of SHV, TEM and CTX β-lactamase genes in Klebsiella pneumoniae isolates was investigated using PCR method and amplification of the desired gene by specific primers. The results of gel electrophoresis of 62 isolates of Klebsiella pneumoniae are given in the figures below. All the isolates that were examined by disk diffusion method to determine the pattern of antibiotic resistance were examined by PCR test using specific primers in terms of the presence of SHV, TEM and CTX genes, and the isolates were respectively (87%) 54 isolates had bla CTX-M gene, (59%) 37 isolates had SHV bla gene, and (98.4%) 61 isolates had bla TEM gene. The results of the examination and the frequency of resistance genes showed that the resistance genes bla SHV, bla CTX-M and bla CTX-M were the most frequent respectively. Gel electrophoresis images are shown in figures (4 to 9).

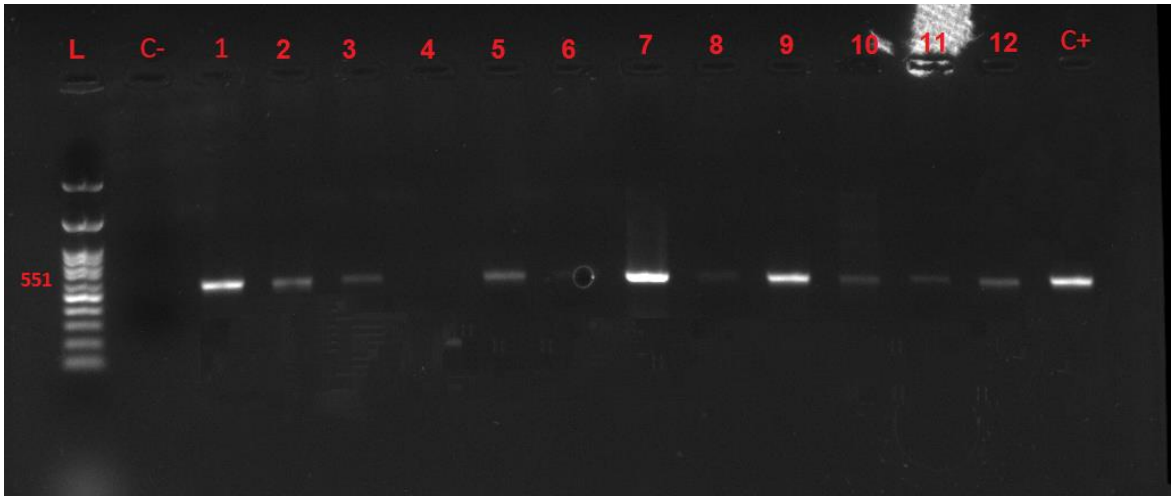

Figure 4. The results of CTX gene electrophoresis of Klebsiella pneumoniae isolates samples 1-12. L: ladder, C-: negative control, C+: positive control.

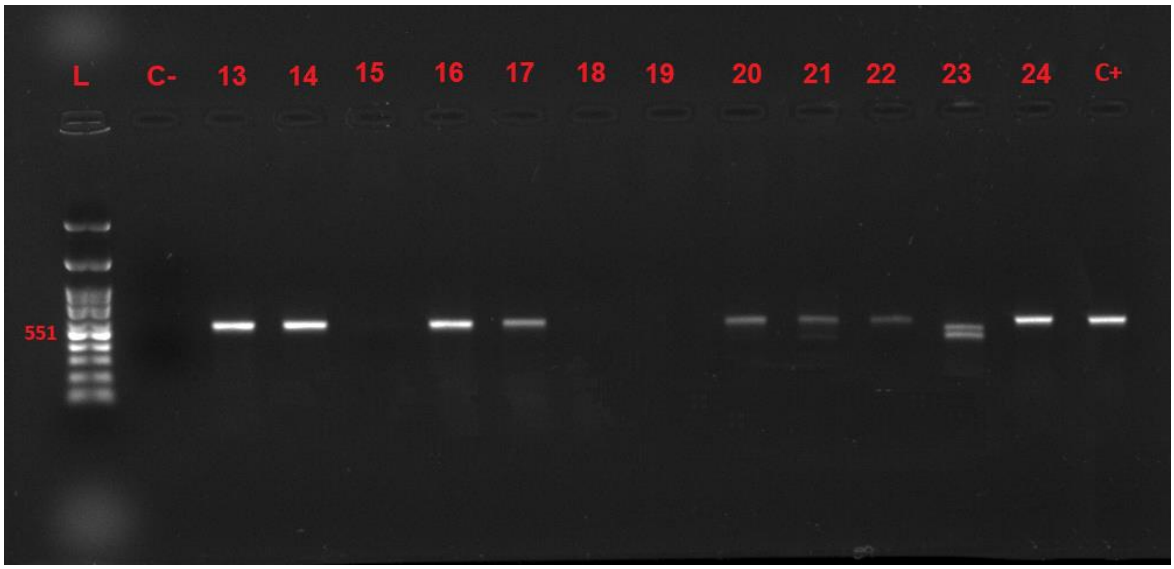

Figure 5. Results of CTX gene electrophoresis of Klebsiella pneumoniae isolates samples 13-24. L: ladder, C-: negative control, C+: positive control.

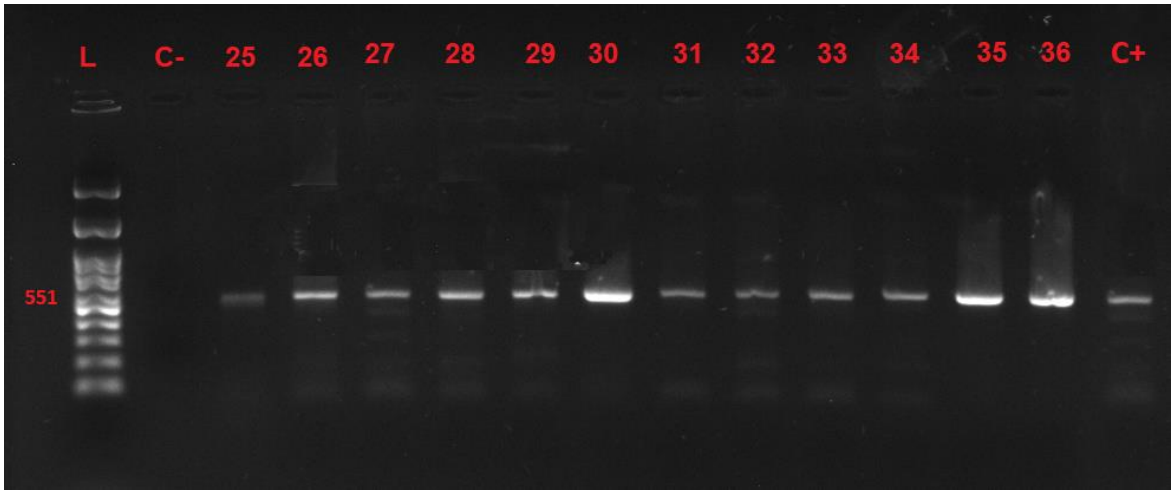

Figure 6. Results of CTX gene electrophoresis of Klebsiella pneumoniae isolates samples 25-36. L: ladder, C-: negative control, C+: positive control

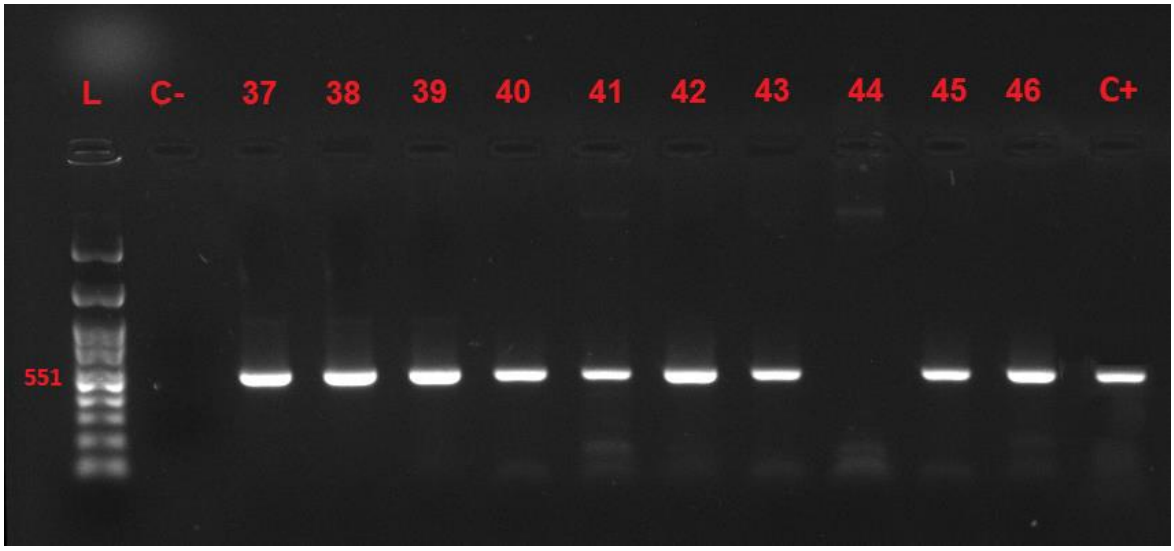

Figure 7. Results of CTX gene electrophoresis of Klebsiella pneumoniae isolates samples 37-46. L: ladder, C-: negative control, C+: positive control

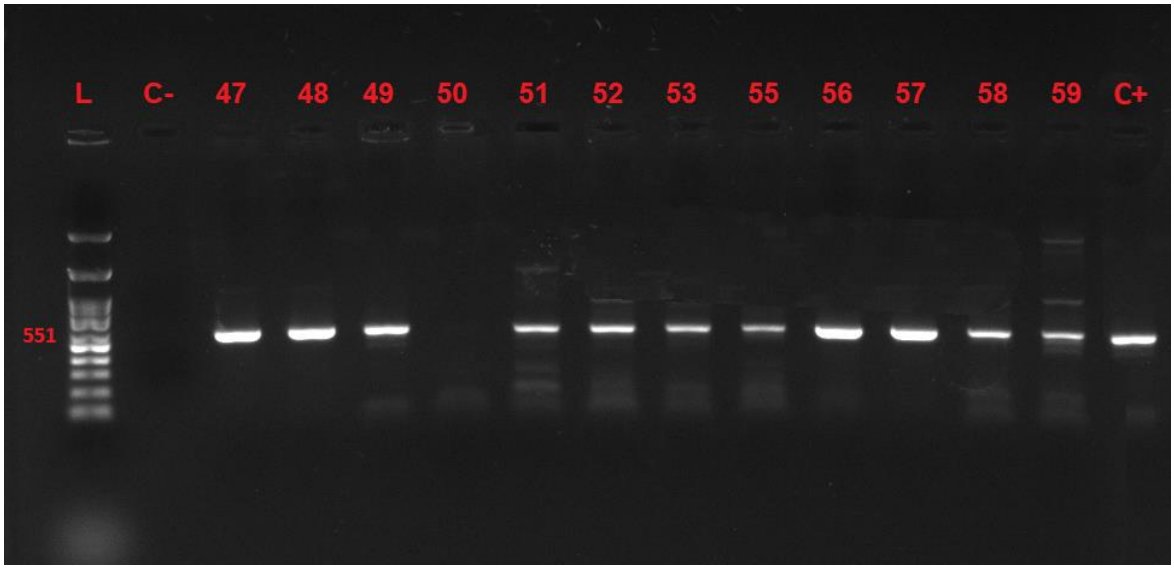

Figure 8. Results of CTX gene electrophoresis of Klebsiella pneumoniae isolates samples 47-59. L: ladder, C-: negative control, C+: positive control

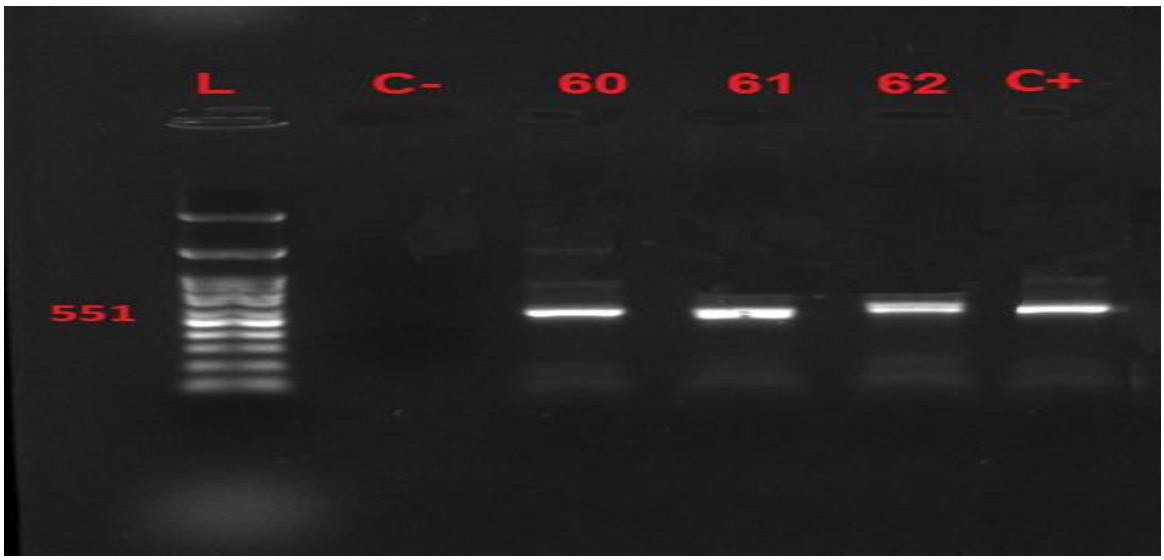

Figure 9. Results of CTX gene electrophoresis of Klebsiella pneumoniae isolates samples 60-62. L: ladder, C-: negative control, C+: positive control

Conclusion

The emergence and spread of Klebsiella broad-spectrum beta-lactamase-producing strains is a serious concern, because the use of some beta-lactam antibiotics is ineffective against these isolates. Examining the antibiotic resistance pattern of isolates producing broad-spectrum beta-lactamases indicates the existence of a high level of antibiotic resistance among these isolates, as in the case of the antibiotics used in this study, such as ampicillin, amoxicillin, and cefixime, we see widespread resistance, while the level of resistance to Antibiotics such as: meropenem are less, which indicates the necessity of optimal use of this generation of antibiotics and prevention of environmental pressure in the direction of turning negative strains into broad-spectrum beta-lactamase producers. The high level of intermediate resistance of the isolates is a serious concern, because they may turn into fully resistant strains with continued use of antibiotics. Due to the importance of the production of ESBLs by organisms and the difficulty of treating the diseases caused by these bacteria, it is necessary to quickly identify the production of ESBLs in isolates by medical diagnostic laboratories based on the simple test recommended by CLSI to confirm the production of ESBLs, which was also done in this research. It should be done for different species of Enterobacteriaceae family. Service laboratories must have plans to control any contamination. Unfortunately, there is a weak cooperation between clinical centers and laboratories in Iran, which should always be mentioned that effective treatment of serious infections cannot be achieved except with close cooperation between medical centers and laboratories. Changing the strategy of antibiotics and using appropriate infection control tools in departments where patients are hospitalized for a long time are important factors that can play a significant role in controlling the spread of ESBLs-producing organisms. Due to the very rapid growth of antibiotic resistance, due to the indiscriminate use of these drugs, there is a need to evaluate the resistance pattern of pathogenic organisms, especially Klebsiella species with CTX, SHV and TEM genes producing ESBL enzymes. Identifying this pattern of antibiotic resistance can be used to prevent the emergence and spread of organism's resistant to multiple drugs.

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