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Determination of the Occurrence of bla SHV gene in Multidrug Resistant Klebsiellapneumoniae isolated from Babylon province

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ABSTRACT
Background and Objective:Klebsiella pneumoniae is an opportunistic pathogen that repeatedly causes various diseases. This bacterium is resistant to many commonly used antibiotics.Multidrug-resistant (MDR) K. pneumoniae produces extended-spectrum β-lactamases (ESBLs), which are a serious issue that needs to be controlled with efficient infection-control techniques. Globally, the prevalence of clinical isolates varies greatly and is evolving rapidly. Therefore, the objectives of this research were to determine the occurrence of thebla SHV1 gene in K.pneumonia isolates and to find the susceptibility of the isolates toβ-lactamaseantibiotics, It shows the pathogenicity of these bacteria in Babylon province and their rate as well as their severity.
Methods:Six hundred clinical samples which included urine, burn, and sputum of patients being treated in Babylon hospitals were collected. The specimens were cultivated on MacConkey agar and eosin methylene blue agar, and then incubated at 37 C° for 24 hr. Morphological features, biochemical tests, and Vitek 2 compact were used to identify the K. pneumoniae isolates. The antibiotic susceptibility tests and the biofilm formation were also performed.
Results:Fifty-six isolates of K. pneumoniae were isolated and identified. The results revealed that the isolates were resistant to cefotaxime, ceftazidime, ampicillin,and imipenem (100%). The lowest resistance of these isolates to antibiotics was tokanamycin (44%), while the biofilm formation rate was (41%). The PCR assay showedthat 52 (93%) of the isolates had the blaSHV gene while four isolates lack this gene.
Conclusion:The emergence of antibiotic resistance in K. pneumoniae is a major global health concern that is an inadvertent result of present medical procedures. Therefore, research into managing multidrug-resistant K. pneumoniae is necessary to lower the incidence and severity of infections.

Keywords: K.pneumoniae, ESBLs, MDR, bla SHV gene ,β-lactamase genes and Babylonprovince

INTRODUCTION

K. pneumoniaeis an opportunistic pathogen that frequently causes nosocomial infections in people with compromised immune systems (1).In 2001, K. pneumoniae strain 11978 was isolated in Turkey and found to be resistant to all β-lactamase antibiotics(2). As doctors, veterinarians and other infection control professionals work to treat and prevent diseases brought on by germs that were formerly believed to be destroyed by antibiotics, the issue of antibiotic resistance has become a burden on their backs(3).Hospitalized patients and antibiotic-resistant opportunistic infections are often caused by this bacterium. It is usually the case that plasmids of E. coli or other members of the enterobacteriaceae family encode the normal bla SHV gene, which is ordinarily encoded on the chromosome of K. pneumoniae. Theories suggest that bla SHV1 separated from the K. pneumoniae chromosome and then extrachromosomally propagated to other bacteria (4).Treatment and management of urinary tract infections caused by K. pneumoniae have become more challenging due to the advent of these bacteria, which display multiple antibiotic resistance phenotypes(5). The most investigated type ESBLs are TEM, SHV, OXA, and CTX-M. There are more than 100 different types of SHV6.β-lactam distribution varies by subtype: Australia, China, and central and south America are home to SHV-type ESBLs, which are mainly found in K. pneumoniae(6). It is generally the case, that plasmids of E. coli or other members of the enterobacteriaceae family encode the regular bla SHV gene, which is normally encoded on the chromosome of K. pneumoniae. According to theories, bla SHV1extrachromosomally spread to other bacteria, after separating from the K. pneumoniae-chromosome (7).The discovery that K. pneumoniae-strains that proliferate as biofilms show a marked rise in intrinsic resistance to antibiotics causes the situation(8). The difference between SHV-2 and SHV-1 was a single amino acid at position 238, where glycine was changed to

serine. SHV-2 was the first ESBL discovered in 1985(9).The existence of ESBLs from the SHV family on self-transmissible plasmids serves as support of the mobility of this class of enzymes. Since the other beta-lactamase has a variable sulfhydryl active site, it is known as SHV. It's becoming widely known that bacteria that produce ESBLs can cause a variety of illnesses, such as intra-abdominal infections, urinary tract infections and cholelithiasis(10).This study was designed to determinetheoccurrence of bla SHV gene in the isolates of multidrug resistant K. pneumoniaeisolated from somehospitals of Babylon province.

MATERIALS AND METHODS

Collection of Samples

A total of 600 samples of both sexes of different ages were collected from some hospitals of Babylon province which included, theImam Sadiq Hospital, Al-MahawilGeneral Hospital, Al-Musayyib General Hospital and Al-Qasim General Hospital. The samples were divided into three groups: the first group included one hundred twenty-four swabs obtained from sputum; the second group included three hundred seventy-six swabs obtained from urine; and the third group included one hundred swabs obtained from burns. These samples were collected during the periodof October 2023 to January 2024.

Isolation and Identification

Every specimen was grown on eosin-methylene-blue-agar and MacConkey-agar, and they all incubated for 24 hours at 37 C°. For additional research, pure colonies were maintained at 4 C° in nutritional broth containing glycerol. Studies were conducted on the size, shape, texture, and colony organization of bacteria cultured on these mediums. A single colony was examined using a 100-x oil-emersion-light-microscope, after being stained with Gram. (11). The Vitek 2 compact, biochemical test, and morphological characteristics (for cells and colonies) were used to identify theisolates.

Antibiotic Susceptibility Test

Using adisk-diffusion-test, the phenotypic detection of ESBLs was completed. K. pneumoniae is a valuable organism for studying the kinetics of induced β-lactamase-synthesis in Gram-negative bacteria, due to its ability to produce β-lactamase with very small doses of β-lactam-drugs.The antibiotics administered included ofloxacin (5µg),ciprofloxacin (5µg), cefotaxime (30µg), ceftazidime (30µg), ampicillin (10 µg), meropenem (10 µg), gentamicin (10 µg), imipenem (10 µg), tobramycin(10µg),chloramphenicol(30µg),kanamycin (30µg), and aztreonam (30 µg).Using the disk diffusiontest, ESBLs was phenotypically identified. Because K. pneumoniae can manufacture β-lactamase with very low levels of β-lactam-medicines, is it an invaluable organism to study the kinetics of induced β-lactamase synthesis in Gram-negative bacteria(12).

Biofilm formation Test

Congo-Red-Dye can create colloidal Complexes. The Complexes that formed at pH-values of 2–3 were all blue, but depending on the ion involved, those that formed at pH-values higher than 12 displayed diverse Hues. Magnesium, for instance, produced a Fuchsia-complex. The adsorption of Congo red on the bacterial cell surface, the ions that predominate at the surface, and the bacterium's production of acid or alkali during the growth are all thought to be responsible for the Congo red reaction in bacteria, which is demonstrated by the coloration of the growth in media containing the dye(13).On Congo red agar which was made up of 1.6 Congo red dye, 10 g of sucrose, and 7.6 g of brain-heart agar powder, the production of biofilm was investigated. On this medium, K. pneumoniae was streakedand incubated at 37 °C for 24 hours. The black color of thecolonies were represented a positive outcome and the red color represented a negative outcome(14)**.**

DNA Extraction

Using a favorgen genomic extraction kit, whole genomic DNA was extracted in accordance with the manufacturer's instructions for K. pneumoniae molecular identification.

PCR primers and Conditions

The K. pneumoniae-specific bla SHV1 genes were detected in this reaction using PCR-cycling-thermal-program settings. Macrogen (Korea) produced the PCR-Primers utilized in this study are shown in Table 1.

Table 1. PCR primers and Conditionstheir used in this study.

primer	Sequence(5----3)	Amplicon Size(bp)	Condition (D,A and E)	Cycle No.	Source

SHV1	F	CTGGGAAACGGAACTGAATG	308	C\1min ˚96 C\ 1 min˚57 C\ 1 min˚72	32	(15)
	R	GGGGTATCCCGCAGATAAAT				

Abbreviations: D, denaturation;F, forward primer; A, annealing; E, extension; R, Reverse primer .

Preparation of Reaction Mixture

As per instructions provided by the company, the reaction mixture was processed using PCR Master Mix (Bioneer, South Korea). The reaction contained 3µL ofDNA Template, , 2µLeach of forward and reverse primer and 6 µL of free nucleases in deionized water, for a total volume of 25 µL. The negative control has all of the previously described data, but it didn't employ a DNA-Template. An automated Thermocycler device was used to carry out the Amplification processes (Clever Scientific, UK) as shown inTable 2.

Table 2. Preparation of PCR reaction mixture

Contents	Volume (µl)
Master Mix	12
DNA Template	3
Forward primer (10 pmol/µl)	2
Reverse primer (10 pmol/µl)	2
Nuclease free water	6
Total volume	25

Agarose Gel Electro

The PCR products were processed for one hour at 75 volts on a 1% agarose gel that had been colored with 5 µL of red safe dye. 5 µL of amplification products plus 1µL of loading dye were loaded to the gel well. The amplified gene electrophoresis fragments were measured using 100–1500 bp DNA marker (Promega, USA) According to Biometra-Germany's gel-documentationssystem, the DNA-Bands was photographed phoresis(16).

Ethical Considerations

All subjects gave their consent and agreed agreement to study the scientific and ethically sound Babylon's Public Health Laboratory and hospitals in Hillah City According todocument with the number B231001 and the date October 10, 2023the studyprotocols, subject information, and form were examined and approved by a local ethics committee of the University of Babylon , collage of Science's Biology Department.

RESULTS

Isolation and Identification

The 56 (referred to as KP1 to KP56) screened isolates of K. pneumoniae used in this research were taken from 600 clinical specimens, including burn, sputum and urine from patients who had the disease. Of these, 39 (10%) were isolated from urine, 8 (8%)were isolated fromburn and 9 (7%) were isolated from sputum as shown in Table3.

Table 3. Distribution of the isolates with their percentages in the collected specimens

Specimen type	Positive for K.pneumoniae		Negative for K. pneumoniae		Total	
	No.	%	No.	%	No.	%
Urine	39	10	337	90	376	100
Sputum	9	7	115	93	124	100
Burn	8	8	92	93	100	100
Total	56	9	544	91	600	100

These isolates were identified according to the morphological characteristics of the coloniesand the cells, biochemical tests, and Vitek 2 Compact. The MacConkey agar colonies were palepink - to red and sticky because they were unable to ferment the sugar lactose.

On eosinmethyl blue, the colonies were sticky pink, they were K. pneumoniaeColonies .It is identified based on the pigments and the odor generation(17).Biochemical characterization of the culture was performed.Negative

results for oxidaseand indole testswere observed, while the isolates showed positive results for catalase, citrate,urease and nitratetests(18).

Table 4: Distribution of growth of K. pneumoniae according to source of isolates ,age ,and local isolates .

Characteristics	Male n=26	Female n=30	p-value
	No. (%)		
Source of isolates			
Urine	16 (61.5)	23 (76.7)	0.371
Burn	4 (15.4)	4 (13.3)	
Sputum	6 (23.1)	3 (10)	
Age (year)			
1-25	8 (30.8)	9 (30)	0.281
26-50	9 (34.6)	12 (40)	
51-75	6 (23.1)	9 (30)	
>76	3 (11.5)	0 (0)	
Local isolates			
Hilla city	13 (50)	16 (53.3)	0.892
Al-Mahaweil	3 (11.5)	5 (16.7)	
Al-Mussayib	4 (15.4)	4 (13.3)	
Al-Qasim	6 (23.1)	5 (16.7)	

Antibiotic susceptibility tests

Fifty-six isolates of K. pnemoniae (KP1 to KP56) the results were evaluated in comparison to 12 favored antibiotics. Most of the isolates exhibited resistance to antibiotics, particularly to medicines that work against β-lactamases. All 56 K. pnemoniae isolates were resistant to cefotaxime, ceftazidime, ampicillin, and imipenem. While the isolates were resistant tochloramphenicol (32%), aztreonam (35%), tobranycin (26%), Kanamycin (44%), gentamcin (14%), meropenem (5%), ciprofloxacin (42%), and ofloxacin (19%).

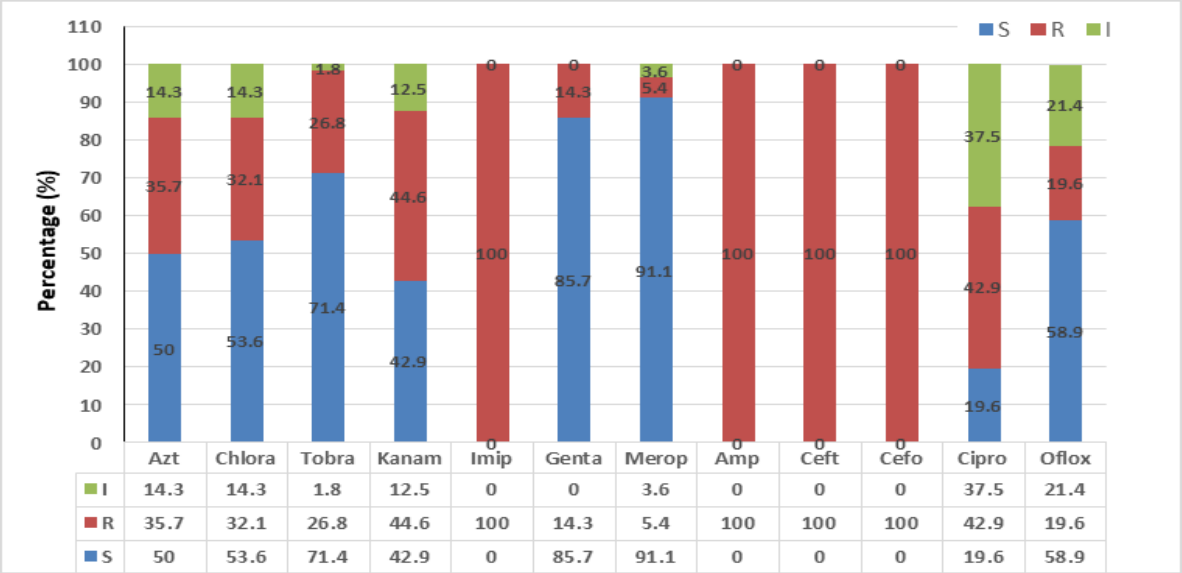

Figure 1: Susceptibility patterns of K. Pnemoniae to various antibiotics used during the present study.Abbreviations: ATM; Aztreonam, C; Chloramphenicol, TOB ; Tobranycin, K ; Kanamycin, IMI ; Imipenem, GN ; Gentamicin, MRP ; Meropenem, AMP ; Ampicillin, CAZ; Ceftazidime,CTX ; Cefotaxime, CIP ; Ciprofloxacin , OFX;Ofloxacin,I;Intermediate ,R;Resistanceand S ;Senstive .

Biofilm formation Test

Biofilm formation was explained and the biofilm capacity of K. pnemoniaewas demonstrated. The results showed that out of 56 isolates, only 23 (41%) isolates (KP1, KP3, KP10, KP13, KP16, KP21, KP22,KP25, KP26, KP27, KP28, KP29, KP30, KP31, KP32, K, P38,KP39, KP48, KP49, KP50,KP53, KP54, andKP55) were positive for biofilm

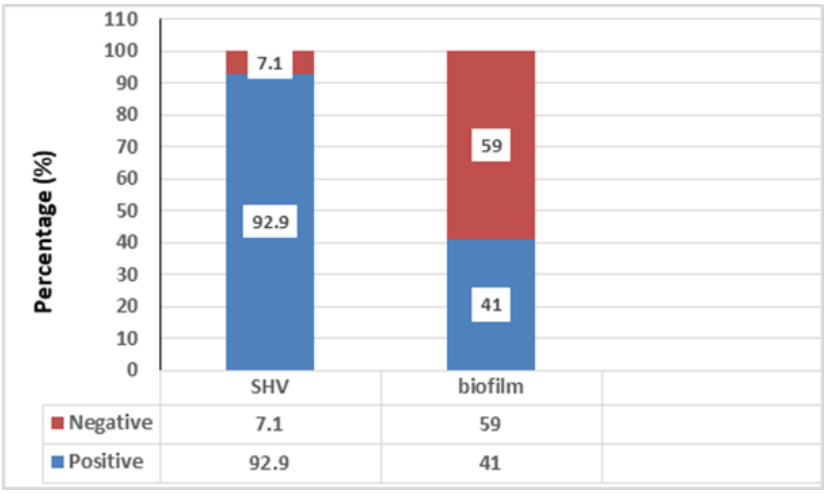

Figure 2: Susceptibility patterns of SHV gene to separate biofilm used for the current study

Table 5 : Distribution of bla SHV gene in K. Pnemoniae isolates.

Isolate No.	SHV gene	Biofilm	Isolate No.	SHV gene	Biofilm
KP1	-ve	+ve	KP29	+ve	+ve
KP2	+ve	-ve	KP30	+ve	+ve
KP3	+ve	+ve	KP31	+ve	+ve
KP4	+ve	-ve	KP32	+ve	+ve
KP5	+ve	-ve	KP33	-ve	-ve
KP6	+ve	-ve	KP34	-ve	-ve
KP7	+ve	-ve	KP35	+ve	-ve
KP8	+ve	-ve	KP36	-ve	-ve
KP9	+ve	-ve	KP37	+ve	-ve
KP10	+ve	+ve	KP38	+ve	+ve
KP11	+ve	-ve	KP39	+ve	+ve
KP12	+ve	-ve	KP40	-ve	-ve
KP13	+ve	+ve	KP41	-ve	-ve
KP14	-ve	-ve	KP42	+ve	-ve
KP15	-ve	-ve	KP43	-ve	-ve
KP16	+ve	+ve	KP44	-ve	-ve
KP17	+ve	-ve	KP45	+ve	-ve
KP18	+ve	-ve	KP46	+ve	-ve
KP19	+ve	-ve	KP47	+ve	-ve
KP20	+ve	-ve	KP48	+ve	+ve
KP21	+ve	+ve	KP49	-ve	+ve
KP22	+ve	+ve	KP50	+ve	+ve
KP23	+ve	-ve	KP51	-ve	-ve
KP24	+ve	-ve	KP52	+ve	-ve
KP25	+ve	+ve	KP53	+ve	+ve
KP26	+ve	+ve	KP54	+ve	+ve
KP27	+ve	+ve	KP55	+ve	+ve
KP28	-ve	+ve	KP56	-ve	-ve

PCR Assay

β-lactamase genes were varied among K. pneumoniae isolates (KP1 to KP56). PCR results and amplification products of β -lactamase genes showed that 52 (93%) of the isolates had the blaSHV gene. While four isolates (KP6, KP33, KP38, and KP49) lack this gene, PCR amplification showed that many K. pneumonia isolates included and lacked β-lactamase genes. Utilizing specific primers, multiplex PCR was performed to determine whether the blaSHV gene was present in MDR isolates. AusDiagnostics Thermal Cyclers (AusDiagnostics, UK) were used for the PCR(19).

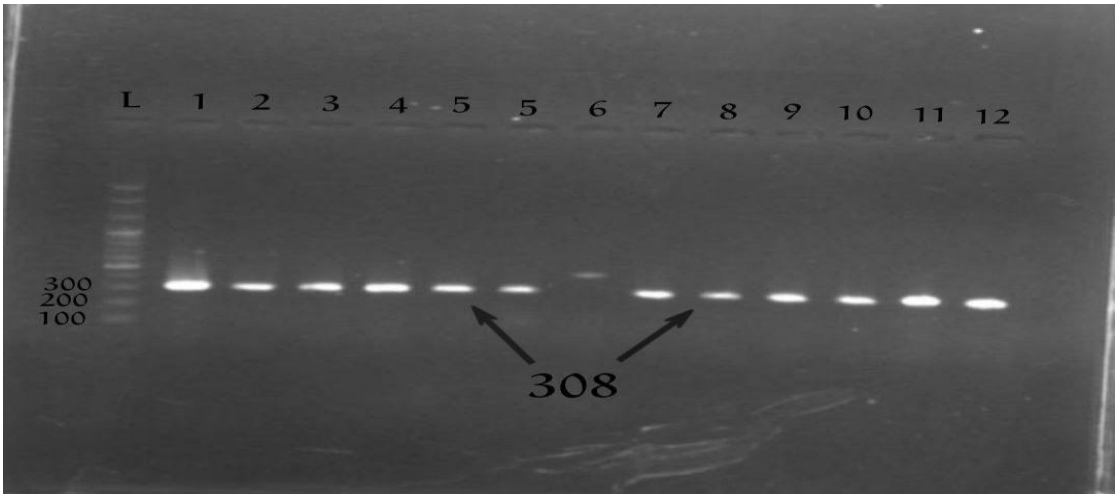

Figure 3: Electrophoresis of blaSHV gene of amplification products from genomic DNA of K. pneumonia isolates on(1 %) agarose gel at 75 v/cm for 60 min. Lane L: ladder, 1.5 Kb; Lanes: 1, 2, 3, 4, 5, 7,8, 9, 10,11, and 12 representthe positive results (308 bp) of the isolates KP1, KP2, KP3, KP4, KP5, KP7,KP8,KP9,KP10,KP11 and KP12.

DISCUSSION

The primary medications for treating infections caused by K. pneumoniae are carbapenems, which include meropenem and imipenem. Regretfully, imipenem-resistance was found to be highly prevalent in this study. The results also showed thatblaSHV gene, was found in some isolates of K. pneumoniae. Therefore, it is better that refrain from prescribing and using antibiotics when they are not necessary to stop their development and spread antibiotic-resistant strains of K. pneumoniae. Thus, MDR K. pneumoniae isolates should be considered a major risk to patients due to its advanced pathogenicity and antimicrobial resistance pattern.blaSHV gene is the most abundant in K.pneumoniae, The result of PCR analysis of this study referred to the presence of a single band (308 bp) gene.ESBLs treating bacterial infections is difficult since germs have become resistant to practically all kinds of antibiotics now in use.

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