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PCR DETECTION OF CTX-M GENES CODING ANTIBIOTIC RESISTANCE IN *Klebsiella* spp. ISOLATES IN AL-NAJAF PROVINCE, IRAQ

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Abstract- One hundred and fifty five of Gram-negative-lactose fermented bacteria grown on MacConkey agar were collected from two main hospitals in Al- Najaf province. The isolates were identified according to cultural characteristics, biochemical activities and Vitick -2 system. 43 (27.7%) isolates were identified as *Klebsiella* spp. Isolates, represented by 35(81.3%) isolates *K. pneumoniae* subsp. *aerogenes*, 4(9.3%) isolates *Klebsiella pneumoniae* subsp. *pneumoniae*, 2 (4.6%) isolates *Klebsiella oxytoca*, as well as one isolate for each of *K. pneumoniae* subsp. *ozaenae* and *K. pneumoniae* subsp. *rhinoscleromatis*. The susceptibility of Klebsiellae isolates to 20 antibiotics were tested, using disc diffusion method; the results have revealed that Klebsiellae isolates were highly resistant to most common antibiotics. The ability of Klebsiellae isolates to produces extended β-lactamase were tested, the results have revealed that 26 (60.4%) isolates produce ESBLs. The ability of Klebsiellae isolates to have *CTX-M* gene was tested; PCR amplification results have shown that 15 of Klebsiellae isolates were possess *CTX-M* lactamase gene.

Keywords- *Klebsiella,* β-lactamase, CTX-M gen

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Introduction

Klebsiella is straight gram-negative rods, usually arranged singly, in pairs or in short chains, non-motile, facultative anaerobic bacteria which has both a respiratory and fermentative type of metabolism. *Klebsiella* spp. exhibit a very mucoid growth due to the large polysaccharide capsule [1].

The normal habitat of this bacteria is the intestinal tract of human and animal, but may be transferred to another site causing a wide range of infections, such as burns, wounds, respiratory tract and urinary tract infections; these infections become difficult to be treated because of the increased ability of Klebsiellae to resist different types of antibiotics [2].

The resistance was mediated by several mechanisms, the important one of which is the production of enzymes encoded by several genes that are carried on some bacterial plasmids, β-lactamase and extended spectrum β-lactamase. Extended spectrum β-lactamases are mostly plasmid-mediated enzymes capable of hydrolyzing and inactivating a wide variety of β-lactam antibiotics, including different types of penicillins and cephalosporines [3,4].

Recently, *Klebsiella* spp. considered as serious pathogen especially in hospital acquired infections because of their ability to resist a wide variety of antibiotics [5]. *K. pneumoniae* is regarded as the highest prevalence of ESBLs followed by *Enterobacter cloacae* and *E. coli.* The cefotaxime resistance was due to enzymes named cefotaximases (*CTX-M*). However, the *CTX-M*s appeared a higher activity against cefotaxime than to ceftazidime [6,7].

The *bla CTX-M* gene variants exhibit less than 40% of identity to *bla SHV* and *bla TEM* [8]. Different properties are involved in the cefotaxime-hydrolysing activity of the *bla CTX-M* genes. The most important amino acids residues for the ESBL activity of the *CTX-M* β-lactamases are Asn104, Ser237, Asp240 and Arg276 [9]. In the late 1990s, seven different *blaCTX-M* genes had been described, but the number of genes have been grown rapidly and now over 80 different variants have been detected [10].

The *CTX-M* enzymes have been detected in a variety of *Enterobacteriaceae* species, from widely separated geographical regions. However, the *CTX-M* variants are mostly detected in *E. coli, S. typhimurium*, *K. pneumoniae* and *Proteus mirabilis* [11]. β-lactamases of *bla CTX-M-1*, *bla CTX-M-9* and *bla CTX-M-14* type are the most common gene types predominant in Europe [12].

The increased prevalence of the isolates-producing ESBL has created an urgent need for laboratory testing methods that will accurately identify the presence of these enzymes in clinical isolates [4]. Many institutes have been published to detect the ESBLs in clinical isolates, including double disk synergy test and combination disk methods, in addition to molecular detection methods including DNA probes, Oligonucleotide typing and gene sequencing have been used to identify the ESBLs. However, the simplest and most com-

mon molecular method has been used to detect the presence of β-lactamases genes is the polymerase chain reaction (PCR) via specific oligonucleotide primers which can be chosen from the sequences available in the public databases such as Genbank [13].

For these reasons, the aim of the study was published to detected the genes encoding β-lactam antibiotic resistance and comparison of genes percentages, via Isolation and identification of *Klebsiella* spp. isolates. Phenotypic detection for β-lactamase enzyme-producing bacterial isolates and detecting the existence of β-lactamase- genes by PCR technique.

Material and Methods

Specimens Collection

One hundred and fifty five Gram-negative lactose fermenting isolates grown on MacConkey agar were collected from two hospital in Najaf province, Alsader Teaching Hospital and Alzahra Teaching Hospital. The isolates are composed of 64 burns infections, 22, 44 and 25 isolates from wounds, urinary tract and respiratory tract infections respectively. From those, 63 isolates from males and 92 from females. The specimens were transferred immediately to the laboratory for culture and identification.

Identification of *Klebsiella* spp. Isolates

The bacterial isolates were identified according to the cultural and biochemical properties. Klebsiellae isolates were distinguished post growth on solid medium. It produces large, smooth, with pink color (lactose fermented), elevated and mucoid colony on MacConkey. Furthermore, the biochemical tests were performed for the identification of Klebsiellae isolates from other isolates. These tests included Indole test, Methyl red test, Voges-proskaur test, Simmon`s Citrate test, Triple Sugar Iron test, Urease test, and Motility test [5].

Antimicrobial Susptibility Test

Disk diffusion method was performed to test the susceptibility of *Klebsiella* spp. isolates to common antibiotics on Mueller-Hinton agar, with an inoculum equal to 0.5 McFarland turbidity according to CLSI [14]. The plates were incubated at 37°C for 18-24 hrs. and the inhibition zone diameters around the antibiotic discs were measured.

Detection of ESBL Producing Isolates

The modified double-disc synergy test (m-DDST) was used to detect the extended spectrum β-lactamase-producing isolates, aztreonam, ceftazidime, cefotaxime and ceftriaxone discs (30 mg) were placed around an amoxicillin-clavulanic acid disc (10 mg) at inter-disc distances (centre to centre) of 20 mm on Muller-Hinton agar inoculated by bacterial suspension equal to 0.5 McFarland, a clear extension of the edge of the aztreonam, ceftazidime, cefotaxime and ceftriaxone discs inhibition zone towards the disc containing clavulanic acid was interpreted as positive for ESBL production [15].

Detection of *CTX-M* β-lactamase Genes (PCR Amplification)

The method descried by Paterson, et al [13] was used to perform PCR amplification. Five µl of bacterial DNA has been used as a template, 40 Pico moles of each primer (Alpha DNA, Canada), 5 µl of 2X Go Taq Green master mix (Promega, USA) (consist of Go Taq DNA polymerase 400 µM of each dNTP, 3 mM MgCl2, reaction buffer (pH=8), yellow and blue loading dyes), and 5 µl of nuclease-free water (Promega, USA), given a total PCR reaction volume of 25 µl. The oligonucleotide PCR primers specific for the β-lactamase genes and PCR products length were

Result

A total of one hundred and fifty five Gram-negative lactose fermented isolates grown on MacConkey agar were collected from two hospitals in Al Najaf province 129 isolates (83.2%) from Al-Sader teaching hospital and 25(16.8%) isolates from AL-Zahra teaching hospital [Table-1]. 92(59.3%) isolates from females and 63(40.7%) from males, the isolates were represented by 64(41.3%) from burns infections and 44(28.4%), 25(16.1%) & 22(14.2%) from urinary tract, respiratory tract and wounds infections respectively [Table-1]. The isolates have been collected from two groups of patients, 105 (67.7%) isolates from hospitalized patients and 50(32.3%) from outpatients. However, 43 isolates have been distinguished as Klebsiellae among the 155 Gram-negative clinical bacterial isolates.

The results revealed that *K. pneumoniae* subspecies *aerogenes* was identified in 35(81.4%) isolates from the total number of *Klebsiella* spp. isolates [Table-2]. *K. pneumoniae* subsp. *pneumoniae* was recorded in 4(9.4%) isolates, whereas *K. pneumoniae* subsp. *ozanae* and subsp. *rhinoscleromatis* were identified in 1 (2.3%) for each one. *K. oxytoca* was recorded in 2(4.6%) isolates from the total number of *Klebsiella* spp.

The results of *Klebsiella* isolates to antibiotic sensitivity have clarified that the Klebsiellae isolates showed high resistance to most common antibiotics of β-lactams, aminoglycosides, tetracyclines, quinolones, sulfonamides and others.

Detection of *CTX-M* β-lactamases Producing Isolates

The determination of ESBLs-producing isolates was performed using double disk synergy test (DDST); the synergism was determined between augmentin (amoxyclav) and members of the third generation cephalosporins (ceftazidime, cefotaxime and ceftriaxone) The results revealed that 26(60.4%) isolates gave positive ESBLs production test, versus 17(39.6%) gave negative results, since the inhibition zone of synergism has been recognized clearly [Fig-1].

Table 1- Distribution of Bacterial Isolates According to Hospitals

Infection	Hospital	Alsader		Alzahra		Total		Total
		Positive	Negative	Positive	Negative	Positive	Negative	
Burns infections	Hospitalized patients	17	47	--	--	17	47	64
	Out patients	--	--	--	--			
Urinary tract infections	Hospitalized patients	1	3	1	2	14	30	44
	Out patients	8	14	4	12			
Respiratory tract infections	Hospitalized patients	3	10	--	4	5	20	25
	Out patients	1	4	1	2			
Wounds infections	Hospitalized patients	7	10	--	--	7	14	22
	Out patients	--	4	--	--			
Total		37	92	6	19	43	112	155
		129		26		155		

Table 2- Distribution of Klebsiella spp. According to Infection Sites

Infection site	Bacteria					Total
	K. pneumoniae subspecies				K. oxytoca	
	K. aerogenes	K. pneumoniae	K. ozaenae	K. rhinoscleromatis		
Burns	15	1	1	--	--	17
Wounds	6	1	--	--	--	7
Urinary tract infection	12	1	--	1	--	14
Respiratory tract infection	2	1	--	--	2	5
Total	35	4	1	1	2	43
%	81.50%	9.40%	2.30%	2.30%	4.60%	

Fig. 1- Double disc synergy test. A/ Positive result of ESBLs production in Klebsiellae, B/ Negative result

Isolates that produce ESBLs were predominant more frequently among burns infections in 11(42.3%) isolates followed by 7(26.9%) of UTI, 5(19.2%) and 3(11.6%) in wound infections and respiratory tract infections respectively [Table-3].

PCR Detection of β-lactamases

Polymerase chain reaction technique has been used to amplify genes encoding the *CTX-M* β-lactamases from genomic DNA of all *Klebsiella* spp. isolates with specific forward and reverse primers; the lengths of amplified genes was 566 bp, The results of β-lactamase genes detection clarify that 15 isolates (57.7%) of ESBLs producers carrying *CTX-M gene* (represented by 1,4,11,14,16,20, 23,27,29,34,35,38,39,42,43 isolates) [Fig-2].

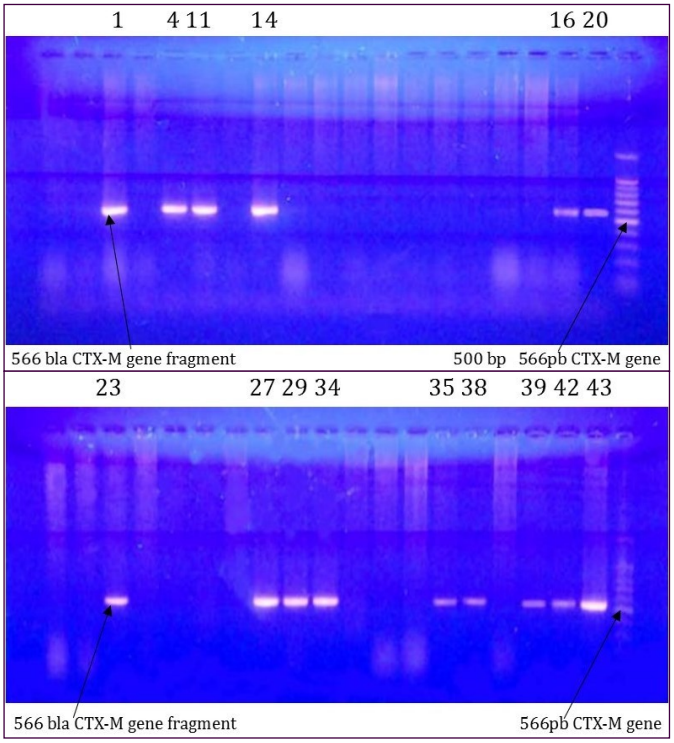

Fig. 2- PCR amplification of CTX-M gene of Klebsiella isolates

Table 3- prevalence of ESBLs- producing Klebsiella spp.

Hospital	Infection								Total = 43	
	Burns		Wounds		Urinary tract infection		Respiratory tract infection			
	Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative
Alsader	11	6	5	2	3	6	3	1	22	15
Alzahra	--	--	--	--	4	1	--	1	4	2
Total (%)	11 (42.30%)	6	5 (19.20%)	2	7 (26.90%)	7	3 (11.60%)	2	26 (60.46%)	17 (39.53%)

Discussion

Klebsiella was very important opportunistic pathogen among gram negative bacilli causing a wide range of infections, such as burns, wounds, respiratory tract and urinary tract infections; these infections become difficult to be treated because of the increased ability of Klebsiellae to resist different types of antibiotics mediated by several mechanisms encoded by several genes that are carried on some bacterial plasmids [2]. For those resean 155 of gram negative isolates were sub cultured on suitable media, (including blood agar and MacConkey agar), and incubated at 37C° for 18-24 hrs., after identification 27.7% of isolates have been identified as *Klebsiellae* spp. among the 155 Gram-negative clinical bacterial isolates. *K. pneumoniae* subspecies *aerogenes* was the most common member of *Klebsiella* spp. causing major infections, it was recorded in 81.3% of *Klebsiella* isolates.

The results of *Klebsiella* isolates to antibiotic sensitivity test have clarified that the Klebsiellae showed high resistance to most common antibiotics of β-lactams, aminoglycosides, tetracyclines, quinolones, sulfonamides and others *Klebsiella* spp. isolates which exhibit resistance to β-lactam antibiotics were suspected to be highly

producers of ESBLs; therefore, all bacterial isolates undergo to ESBLs production test. The isolates that produce ESBLs were predominant among *Klebsiella* and they were more frequently among burns infections.

However, the detection rate of ESBLs-producing Klebsiellae isolated from clinical samples differ from each other. Screening for the existence of ESBLs among *K. pneumoniae* carried out in study of Taneja, et al [15], he reported that the highest rate of ESBLs production was found to be in *Klebsiella* spp. (51.2%), followed by *E. coli* (40.2%), *Enterobacter aerogenes* (33.4%) and *pseudomonas aeroginosa* (27.9%) isolates.

The incidence of ESBLs-producing strains among clinical isolates of *Klebsiella pneumoniae* have been on steady increase during few years ago, thus accounts about 17% of all nosocomial urinary tract infections [16].

PCR Detection of β-lactamase (CTX-M)

The genomic of *Klebsiella* was tested to determinant the CTX-M genes, using PCR technique with specific forward and reverse primer, 57.7% of β-lactamase producing isolates were caring CTX-M gene in their genomic DNA. The findings of the present study support the hypothesis that *CTX-M* gene is emerging as the dominant ESBL type in clinical isolates, these findings were agreement with the findings recorded by [13]. All *CTX-M* enzymes in *Klebsiella pneumoniae* and *E. coli* belonged to the *CTX-M-1* as illustrated by restriction analysis [17].

In a multi-centric study from Russia, *CTX-M* gene was reported in 35.9% of *E. coli* and 34.9% of *K. pneumoniae* ESBLs isolates [18]. [19] reported 87% prevalence of *CTX-M* enzyme among ESBLs producing Klebsiellae in a tertiary care hospital of Greek.

Conclusion

Kebsiella pneumonia subsp. *aerugenes* was predominant in most of *Klebsiella* infections, the *Klebsiella* isolates was highly resistance for most of antibiotics and the CTX-M was predominant in *Klebsiella* isolates.

Conflict of Interest : None declared.

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