Detection of TEM and SHV genes in *Escherichia coli* and *Klebseilla* species isolated from cancer patients in Al-Diwaniya Governorate

Adnan H. Al-Hamadani*,Adel M. Al-Rikabi** and Atheer F. Al-Fatlawi

الخلاصة

الكشف عن الجينات المشفرة لبعض إنزيمات البيتا-لاكتاميز واسعة الطيف في بكتيريا إشيريشيا القولون وأنواع الكليبسيلا المعزولة من مرضى سرطان القولون ومرضى سرطان المثانة باستخدام الطرق المظهرية والطرق الجزيئية.

جُمِعت 61 عينة براز وإدرار من 61 مريض مشخصين سريريا بصورة قطعية كمصابين بالسرطان. شخصت العينات بالطرق الاعتيادية المظهرية والاختبارات الكيموحيوية وكذلك باستخدام نظام الفايتك. اجري فحص الحساسية للعزلات المدروسة باستخدام طريقة انتشار القرص.تم التحري عن انتاج انزيمات البيتا-لاكتاميز واسعة الطيف بطريقة تآزر القرص المزدوج بيمنا تم التحري عن وجود جينات هذه الانزيمات باستخدام تقنية تفاعل السلسلة المتبلمرة (PCR).

أظهرت الدراسة بان نسبة بكتيريا إيشريشا القولون وأنواع الكليبسيلا مجتمعتين في عينات البراز كانت (73 %) أي 19 من أصل 26 عينة, أما في عينات الإدرار فقد كانت نسبتهما (73.9 %) أي 17 من أصل 23 عينة. أظهرت نتائج المسح الأولي لمقاومة مضادي البيتا-لاكتام (الامبسيلين والاموكسيسيلين) بان الغالبية العظمى من العزلات كانت مقاومة لهذين المضادين وبنسبة 19 (86.4 %) لبكتيريا ايشريشيا القولون و 13 (92.8 %) لبكتيريا الكليبسيلا وقد أظهرت النتائج بأن جميع العزلات كانت مقاومة على الأقل لثلاث أصناف من المضادات التي تم اختبارها لذلك أُعتبرت هذه العزلات متعددة المقاومة. اظهر الاختبار التوكيدي باستخدام طريقة تآزر القرص المزدوج إن 9 عزلات فقط من مجموع 32 من العزلات المختبرة كانت منتجة فعلا لإنزيمات البيتا-لاكتاميز واسعة الطيف. أظهرت نتائج التشخيص الجزيئي لبعض جينات البيتا-لاكتاميز واسعة الطيف (bla_{TEM} و bla_{SHV}) باستخدام تقنية تفاعل البلمرة المتسلسل بان جميع العزلات المختبرة كانت تحمل على الأقل واحدا من الجـينات المذكورة أعلاه, وكان جين (SHV) بنسبة (66.7 %) ثم جين (TEM) وبنسبة (55.6 %).

بينت الدراسة تواجد عالي لجيني البيتالاكتاميز واسعة الطيف اللذان تم التحري عنهما في العزلات السريرية لمرضى سرطان القولون وسرطان المثانة.

Abstract

The aim: Detection of some genes that encode to some extended spectrum beta-lactamase enzymes in *E. coli* and *Klebseilla* spp. isolates from colon and bladder cancer patients by using the phenotypic and genotypic method.

* Department of Microbiology/College of Medicine/Al-Qadissiya University

** Department of Surgery/College of Medicine/Al-Qadissiya University

Methods: A total of 61 stool and urine samples collected from 61 patients definitely and clinically diagnosed with cancer. All these isolates were identified by conventional methods and confirmed by VITEK-2 system. Antimicrobial susceptibility testing was determined using disk diffusion methods. Investigation of production of ESBL was done by DDST methods while screening of β-lactamase genes was done by PCR technique.
Results: The study revealed that *Klebsiella* species and *E. coli* were detected in 17 (73.9 %) from urine samples, and 19 (73 %) from stool samples, the vast majority of isolates were found to be resistant to ß-lactam antibiotics (ampicillin and amoxicillin) in the primary screening test at percentage 19 (86.4 %) of *E. coli* isolates and 13 (92.8 %) *Klebsiella* spp. and all the tested isolates are resistant to a minimum of three classes of antibiotics to which they are tested, hence the isolates are considered to be multidrug resistant. the confirmative detection of ESBL by double disk synergy test showed that out of 32 β-lactam resistant *E. coli* and *K. pneumoniae* subsp. *pneumoniae* examined in this study, ESBLs were detected in 9 (28.1 %) isolates. The results of molecular detection of ESBL genes *(bla*$_{TEM}$ and *bla*$_{SHV}$) by using PCR technique showed that all the tested ESBL producing isolates were carried at least one of the ESBL genes SHV and (66.7 %), TEM (55.6 %).
Conclusion: There is a high occurrence of the tow ESBL genes in clinical isolates of colon and bladder cancer patients.

Introduction

In spite of many potent and broad-spectrum antibiotics had been marketed in the past decades, bacterial infections still cause substantial mortality and morbidity among cancer patients. This may be related to more immunosuppressant medications and aggressive diagnostic or therapeutic tools in clinical management of various cancers.[1] Bacterial infections associated with multidrug resistance have been implicated in high mortality and morbidity reported among cancer patients.[2] Gram-negative bacilli could cause severe sepsis and mortality, especially *Escherichia coli* and

Klebsiella pneumonia that remain the prevalent causes of bacterial infections in cancer patients.[3][4]

The resistance is 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. ESBL are mostly plasmid-mediated enzymes capable of hydrolyzing and inactivating a wide variety of β-lactam antibiotics, including different types of penicillins and cephalosporines.[6] ESBLs have emerged as an important mechanism of resistance to β-lactam antibiotics in Gram negative bacteria, mostly in Enterobacteriaceae.[5]

The recognized risk factors for acquisition of ESBL-producing pathogens included previous antibiotics use, longer hospital stay, and intravascular devices, which were also characteristic in cancer patients.[7][8] Resistance to the extended-spectrum cephalosporins can occur in *E. coli* and *Klebsiella* spp. via the production of β-lactamases that are capable of hydrolyzing the oxyimino-cephalosporins and monobactams. So these organisms become uniformly resistant to oxymino- β-lactam antibiotics.[9]

For this reason, this study was designed to detect some genes that encode beta-lactamase mediated resistance in *E. coli* and *Klebseilla* spp. isolates from patients with cancer of colon and urinary bladder by using the phenotypic and genotypic method.

Methodology

Sample collection: Sixty one patients whom definitely clinically diagnosed with cancer were included and attended to Al-Diwaniya Teaching Hospital. In this study a total of 33 urine specimens were taken from 33 bladder cancer patient and 28 stool specimens from 28 colon cancer patients plated onto MacConkey agar and blood agar and incubated aerobically at 37˚C overnight. All samples were investigated for the presence of *Escherichia coli* and *Klebsiella* spp.

Isolates identification: Bacterial isolates were identified to the level of species using the traditional morphological and biochemical diagnostic tests. All *E. coli* and *Klebsiella* spp. Isolates

were confirmatively diagnosed by VITEK2 system by using VITEK®2 GN kit, then stored at maintenance medium until further tests.

Primary screening of β-lactam resistantance: Isolates of *E. coli* and *Klebsiella* spp. were screened on Muller-Hinton agar supplemented with ampicillin and amoxicillin (each alone) at final concentrations of 50 and100 µg/ml, respectively.

Antimicrobial susceptibility testing: The isolates were screened for their antibiotic resistance against 18 antimicrobial agents of different classes using Kirby-Bauer disk diffusion method [14] and interpreted according to the CLSI.[10]

Detection of ESBLs by disk approximation method: Cefotaxime, Ceftazidime, Ceftriaxone, and Aztreonam antibiotic discs were placed on Muller Hinton agar at same distances (30 mm from center to center) from the Amoxiclav disc (which placed in the center of the plate). A clear enhancement of the zone of inhibition on sides of centric disc toward other discs mean that these bacteria are ESBL producers.[11]

Molecular detection of ESBL genes by PCR: DNA preparation from ESBL producer isolates was performed by salting out method. The plasmid DNA used as a template for the detection of bla_{TEM} and bla_{SHV} genes by using four specific primers Forward TEM DNA sequence AAACGCTGGTGAAAGTA and reverse TEM DNA sequence (5'-3') AGCGATCTGTCTAT at product size bp 822, forward SHV DNA sequence (5'-3') ATGCGTTATATTCGCCTGTG and reverse SHV DNA sequence (5'-3') TGCTTTGTTATTCGGGCCAA at product size bp 753.

Amplification reaction mixture contained: 5µl of DNA template, 2µl of 10 pmole\µl of each primer (upstream and downstream), 25 µl of AccuPower PCR PreMix, and 16 µl of nuclease free water. The reaction was done under following condition of thermocycler: Predenaturation94˚ C 30 sec. Cycles35 Cycles denaturation94˚ C 30 sec Annealing45˚ C 1 min. 60˚ C 1 min Extension. 72˚ C 1 min. Final extension72˚ C 10 min. Final hold step 4˚ C

Statistical analysis: The results were analyzed statistically by Chi-square (X^2) test at the level of significant when P-value ≤ 0.01 [12]

Results and Discussion

Isolation and identification

Escherichia coli and *Klebsiella* spp. were detected in 17 (73.9 %) of the urine samples, while they were 19 (73 %) from stool sample, in additions to other bacterial isolates. It was clear from table (1) that the *E. coli* isolates were the most common pathogens isolated from both bladder and colon cancer patients (44.9 %). This results were previously indicated by many researchers such as who isolated *E. coli* (40 %) from urine of kidney cancer patients and (50 %) from stool of colon cancer patients. [13] In Najaf City, *E. coli* were isolated at percentage (42.6%) from urine samples with significant bacteriuria patient. *E. coli* were isolated from patients with different cancers at (44 %) and (56.3 %) respectively. [14][15]
On the other hand, *E. coli* was the most common organism isolated from patients suffering from cancer in urinary tract[16], and it were (34.5%) from urine of bladder cancer patients.[17] The cause of high incidence infection with *E coli* belongs to the fact that these bacteria leave their natural place (micro flora of intestine) to urinary pathways causing inflammation of urinary tract.[18] The ability of uropathogenic *E. coli* to cause UTI is related to general virulence factors such as α-hemolysin together with pili-mediated adherence to uroepithelial cells.[29] The possibility of getting infection increases in immune compromised patients as patients with cancer, especially when they take anti cancer drugs.[19]

Table (1): Distribution of bacterial species in cancer patient's samples.

Bacterial isolate	No. of isolates (%)		Total (%)
	Urine	Stool	
Escherichia coli	9 (39.2 %)	13 (50.0 %)	22 (44.9 %)
Klebsiella pneumoniae	6 (26.1 %)	5 (19.2 %)	11 (22.4 %)
Klebsiella oxytoca	2 (8.7 %)	1 (3.8 %)	3 (6.1 %)
***Proteus* spp.**	3 (13 %)	3 (11.5 %)	6 (12.2 %)
***Enterobacter* spp.**	1 (4.3 %)	4 (15.4 %)	5 (10.2 %)
Pseudomonas aeruginosa	2 (8.7 %)	0 (0.0 %)	2 (4.1 %)
Total	23 (100 %)	26 (100 %)	49 (100 %)

Klebsiella spp. come in second stage after *E. coli* in our study results at percentage (28.6 %) which previously mentioned by

local study reported that *Klebsiella* spp. isolates percentage was (23.1%) in urine sample from patients with significant bacteriuria.[18] Another study showed that lower ratio of *Klebsiella* spp. in urine of cancer patient.[23] Moreover, many reports refered that *K. pneumoniae* was the prevalent bacterial species in significant bacteriuria[24].

Klebsiella pneumoniae is considered the micro flora of intestine; they pose important virulence factors as capsule helping in increasing the opportunity to infect urinary system. The capsule protects the bacteria from harsh conditions and increases their resistance to immune system as phagocytosis process.[32]

Primary screening of β-lactam resistant isolates:

The results of present study showed that 19 (86.4 %) of *E. coli* isolates were resistant to both ampicillin and amoxicillin (Table 2). All these isolates were able to grow normally in the final concentrations of 50-100 µg/ml of these two antibiotics. This result was closely similar with local study results which showed that (82.6 %) *E. coli* that recovered from urine samples were resistant to these two antibiotics.[20] But it differed with that obtained by other local studies who reported that all the clinical isolates of *E. coli* were resistant to ampicillin and amoxicillin in Hilla.[25] However this results was higher than that obtained by [26] who found that *E. coli* was the principal pathogen isolated from patients with UTI with high susceptibility to ampicillin (72.6%), but was lower than that reported in Korea by [4] who found that (91%) of *E. coli* isolates were resistant to ampicillin. The reason of β-lactam resistance of *E. coli* isolates is probably due to the production of TEM β-lactamases, which may be genetically localized on the chromosome or on a plasmid. The TEM-1 is the most commonly encountered β-lactamase in Gram-negative bacteria; up to 90% of ampicillin resistance in *E. coli* is due to the production of TEM-1.[27]

Table (2): β-lactam resistance of *E. coli* and *Klebsiella* spp. Isolates

Isolate	No. of isolates	No. (%) resistant isolates
Escherichia coli	22	19 (86.4 %)
***Klebsiella* spp.**	14	13 (92.8 %)
Total	36	32 (88.8 %)
Cal. X^2 = 0.365 tab. X^2 = 0.004 df = 1 P-value = 0.949		

The present results also showed that 13 (92.8 %) *Klebsiella* spp. isolates were resistant to both ampicillin and amoxicillin. The statistical analysis showed a significance differences ($P \leq 0.01$) among tested isolates. This relatively high ratio is similar to some local studies ratio showed that all (100 %) *Klebsiella* isolates were resistant to both ampicillin and amoxicillin,[28] Antibiotic resistance arises quickly and spreads rapidly, especially when resistance genes are horizontally transferred via plasmids and integrons among individuals, among species, and even among bacterial kingdom.[29]

The antibiotic susceptibility pattern:

In this study, all the (32) β-lactam resistant *E. coli* (n=19) and *Klebsiella* spp. (n=13) isolates were screened for their antibiotic resistance against 18 antimicrobial agents of different classes using Kirby-Bauer disk diffusion method. A strain is considered a multidrug resistant (MDR) if an isolate is resistant to representatives of three or more classes of antibiotics.[30] In the present study, all the tested isolates are resistant to a minimum of three classes of antibiotics to which they are tested. Hence the isolates are considered to be multidrug resistant. Similar results with MDR isolates have been reported with other authors in Iraq, found that 56.8% of clinical *E. coli* isolates in Najaf were resistant to more than five antimicrobial agents. [31] others revealed that all *Klebsiella* isolates were found to be resistant to at least 8 antibiotics tested.[32] Hence all the isolates were considered to be multidrug resistants

Table (3): The antibiotic susceptibility of *E. coli* and *Klebsiella* spp. Isolates

Type of antibiotic	No. (%) of Resistant *E. coli* (n = 19)	No. (%) of Resistant *Klebsiella* spp. (n = 13)
Amikacin	1 (5.3 %)	1 (7.69 %)
Amoxicillin- Clavulanate	16 (84.21 %)	12 (92.3 %)
Azteronam	13 (68.4 %)	8 (61.5 %)
Cefotaxime	14 (73.7 %)	7 (53.8 %)
Cefoxitin	8 (42 %)	5 (38.5 %)
Ceftazidime	5 (31.6 %)	6 (46.1 %)
Ceftriaxone	14 (73.7 %)	8 (61.5 %)
Chloramphenicol	11 (57.9 %)	1 (7.69 %)
Cifixime	10 (52.6 %)	9 (69.2 %)
Cefepime	15(78.9%)	9 (47.4 %)
Ciprofloxacin	11 (57.9 %)	0 (0.0 %)
Co-trimoxazole	9 (47.4 %)	8 (61.5 %)
Gentamycin	9 (47.4 %)	10 (76.9 %)
Nalidixic acid	8 (42.1 %)	3 (23.1 %)
Pipracillin	17(89.5 %)	11 (84.6 %)
Rifampine	7 (36.8 %)	10 (76.9 %)
Tetracycline	10 (52.6%)	9 (69.2%)
Trimethoprim	8 (42.1%)	8 (61.5 %)

Disk Approximation Method:

The results of present study showed that out of 32 β-lactam resistant *E. coli* and *K. pneumoniae* subsp. *pneumoniae* examined in this study, ESBLs were detected in 9 (28.1 %) isolates (Table 4). They are distributed as 4 (21 %) isolates belonging to *E. coli* and 5 (38.5 %) isolates belonging to *K. pneumoniae* subsp. *pneumoniae*. However, results showed that the frequency of ESBL-producing isolates was higher than that reported by other researchers who found that only 4 *Klebsiella* isolates (10.5 %) were identified as ESBL-producers by using disk approximation method.[32] In another local study, ESBLs were detected in 11 isolates (18.3%) out of 60 ß-lactam resistant *E. coli* and *K. pneumoniae subsp. Pneumoniae* isolates.[22]

Table (4): Frequency of ESBL production in *E. coli* and *Klebsiella* spp. isolates by disk approximation

Type of isolate	No. of organism	No. of ESBL producers
E. coli	19	4 (21.0 %)
K. pneumoniae subsp. *pneumoniae*.	13	5(38.5 %)
Total	**32**	**9 (28.1 %)**
Cal. X^2 = 1.157 **tab X^2 = 0.456**	**df = 1**	**P-value = 0.282**

But it was lower than reported in Hilla city, out of 15 ß-lactamase-producing Enterobacteriacea isolates; only 7 isolates (46.7%) were detected as ESBL-producers by using disk approximation method,[34] in Kuwait, ESBL producing *E. coli* was 62% [33]; in Iran, only 16.8% was ESBL producers.[34] In Baghdad, only 8 (11.1 %) isolates of gram negative bacteria isolated from cancer patients were ESBL producers. [16] On the other hand, the isolation rate were observed in Korea[35], in Latin America[36] , and in South India [37] were 9.3%, 8.5%, and 8.3%, respectively.

As shown in table (4) and Figure (1), only 5 (38.5 %) out of 13 *Klebsiella* spp. isolates were confirmed as ESBL producers. However, in India, 66.7% of *Klebsiella* spp., isolates were identified as ESBL producers; [38] and in Kuwait, ESBL production was detected in 82.1% of the *K. pneumoniae* isolates.[33] In this study, the proportion of ESBL producers is considered low when compared with the results of initial screen disc test, which all these isolates were cefoxitin resistant, and any isolate was cefoxitin resistant indicates that it is possibly AmpC β-lactamase producers which can mask ESBL production in the standard CLSI ESBL confirmatory tests. False results are supposed to occur if the AmpC activity is larger than activity of ESBL which may lead to failure treatment.[39][40]

Therefore, it can be said that these isolates may have ESBL enzymes, but they can't be detectable by third generation cephalosporins with amoxicillin/clavulanic acid may be due to the existence AmpC enzymes which act as a mask against the production of ESBL enzymes in confirmatory tests. Unlike ESBLs, AmpC β-lactamases do not confer resistance to fourth-generation cephalosporins. Therefore, the use of fourth generation

cephalosporins, should facilitate the detection of ESBLs in organisms that also produce AmpC β-lactamases.[11][41]

On the other hand, ESBL producing isolates don't always show *in vitro*, because the cases may show that especially simultaneous presence of metallo-enzymes with carbapenem hydrolyzing activity, [42] extended spectrum oxacillinases (e.g. OXA-10), effective of GES-2 on clavulanic acid, [43] or may combine mechanisms of resistance like efflux pumping and impermeability.[44] Many researchers mentioned that ESBL enzymes is difficult to detect phenotypically with the existing AmpC β-lactamase genes.[45][46][47] By the way, infections caused by ESBL- and AmpC β-lactamase-producing Gram-negative bacteria complicate therapy and limit treatment options.[48]

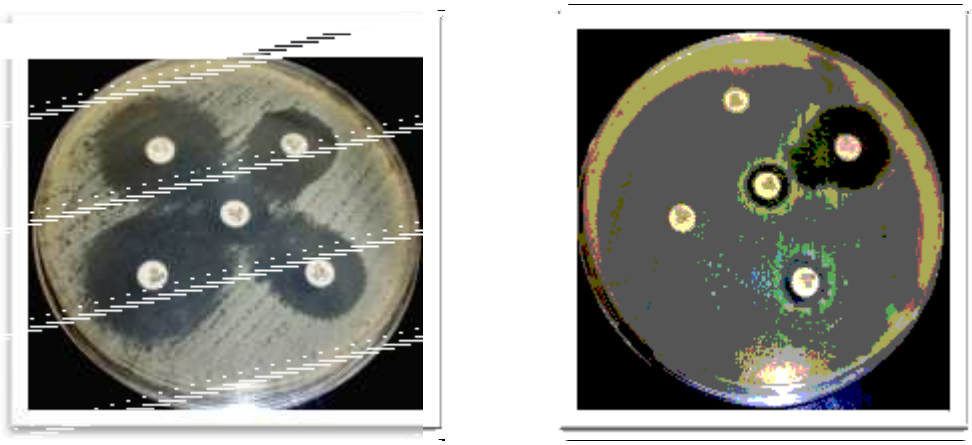

Figure (1): Disk Approximation Test for detection of ESBL in K. pneumoniae subsp. pneumonia and E. coli

The overall ESBL production rate for *Enterobacteriaceae* was 10.5%; the highest rates were encountered in Egypt (38.5%) and Greece (27.4%) and lowest in the Netherlands (2%) and Germany (2.6%).[50] It was found that in many parts of the world almost 10- 40% of strains of *E. coli* and *K. pneumoniae* carry genes encoding ESBLs.[9]

Molecular Detection of ESBL Genes by PCR:

Results showed that, 2 (50 %) out of 4 *E. coli* isolates were able to yield amplification products with TEM-PCR specific primers figure (2, A), this similar to local study in Najaf, 14 (66.6%) out of 21 *E. coli* isolates were able to show TEM gene [20], but disagree with another local

study in Hilla, reported that only 1 (20 %) out of 5 of *E. coli* isolates was possess TEM gene[25] and also disagree with study in Najaf show that 5 (25%) of *E. coli* can revealed show TEM gene. [40]

Table (5): The percentages of *bla*$_{TEM}$ and *bla*$_{SHV, M}$ genes in ESBL positive *E. coli* and *Klebsiella* spp. isolates.

Type of Isolate	No.	TEM	SHV
E. coli	4	2 (50 %)	3 (75 %)
K. pneumonia* subsp. *pneumonia	5	3 (60 %)	2 (40 %)
Total	9	5 (55.6 %)	5 (55.6 %)

TEM-type ESBLs are the first plasmid-mediated ß-lactamase that is often found in genera of Enterobacteriaceae such as *E. coli*, *K. pneumoniae*, and *Proteus mirabilis*;[51] The native TEM-1 β-lactamase confers resistance to ampicillin, penicillin and first-generation cephalosporins such as cephalothin. This enzyme is responsible for 90% of ampicillin-resistance in *E. coli* isolates.[27]

The present study showed that 3 (60 %) out of 5 isolates of ESBL producer *Klebsiella* spp. were able to yield amplification products with TEM-PCR specific primers (2) which in contrary those reported by local investigation show only 2 (15.4%) of *K. pneumoniae* subsp. *pneumoniae* gave PCR products with TEM-specific primers,[20] but agree with study in Hilla who found that 7(77.8 %) out of 9 isolates *Klebsiella* spp. were able to yield amplification products with TEM. [25] The TEM β-lactamases spread worldwide and it is known to be found in many Enterobacteriaceae.

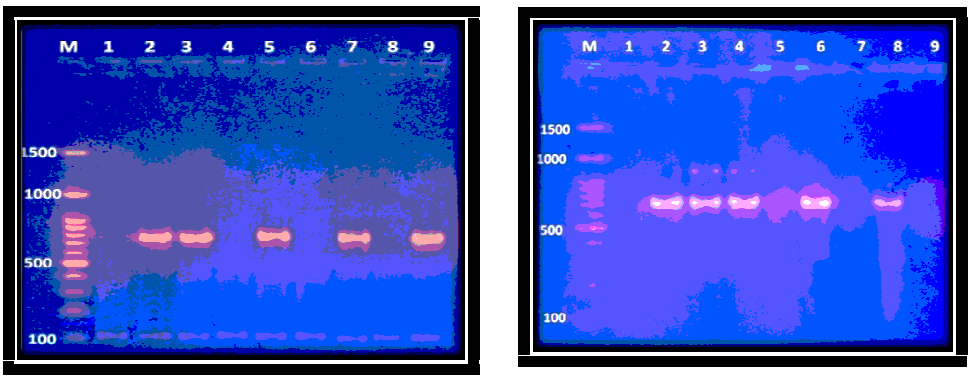

Figure (2): Ethidium bromide stained agarose gel showing PCR amplification products *E. coli* and *K. pneumonia* subsp. *pneumonia*.
A: TEM (822 bp) primers B: SHV (753 bp) primers

However, *Klebsiella* spp. shows reduced susceptibility to first and second generation cephalosporins by the production of plasmid-mediated, TEM β-lactamase. Since 1980s, the emergence of resistance to third generation cephalosporins has been reported in strains of *K. pneumoniae*.[52]Another study reported that 4 (10.2%) of 39 *K. pneumoniae* isolates were positive to TEM enzymes. [53] The first ESBLs observed at the teaching hospitals of Clermont-Ferrand, France, in July 1984, the cefotaximase TEM-3/CTX-1 was produced by *K. pneumoniae* .[54] In the United States, the enzymes which occur commonly in outbreak caused by *K. pneumoniae* are TEM-10, TEM-12, and TEM-26.[55]

The present study found that 3 (75 %) out of the *E. coli* 4 ESBL producer were yield amplification products with SHV-PCR specific primers (Table 5). This rate is contrary with locl study who found that 3 (14.3%) out of the 21 β-lactamase-producing *E. coli* were positive by PCR for bla_{SHV} gene. [20] In Najaf, local study showed that 6 (30 %) out of 20 of *E. coli* can reveal show SHV gene. [40]

The majority of SHV enzymes are found in strains of *K. pneumoniae*. Nevertheless, these enzymes have also been found in *E.coli*.[56] It was recently reported that 15.1% of *E. coli* isolates from clinical samples in Turkey were able to produce SHV enzymes depending on PCR test.[57] Study from Iran found that the frequency of *bla*TEM genes among the ESBL Gram-negative isolates were 9.0%.[58] While the present result was lower than other studies in other parts of the world, in Germany, 70% of *E. coli* and *Klebsiella* spp. had *bla* TEM genes,[41] in Thailand, *bla*TEM genes were 78% of the confirmed ESBL producers.[59]Table (5) also demonstrate that 2 (40 %) of 5 *Klebsiella spp.* were able to produce SHV enzymes, similar to [20] who showed that 7 (53.8%) out of the 13 β-lactamase-producing *K. pneumoniae* subsp. *pneumoniae* isolates yield amplification products with SHV-PCR specific primers. While another study reported that the rate were 8 out of 9 of *Klebsiella spp.* isolates.[25] *Klebsiellae* generally have class-A chromosomal β-lactamase, which differ greatly from the class-C types (AmpC types). Most *K. pneumoniae* isolates have chromosomally or plasmid-mediated SHV-1 β-lactamase, which is a narrow-spectrum β-lactamase with activity against penicillins.[60] More than 50 variants of SHV which are important worldwide and currently recognized on the basis of unique combination of amino acid replacement.[61]

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