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# Molecular Study of Extended Spectrum Β-Lactamases Genes (Blashv, Blactxm) in Klebsiella Pneumoniae Isolated from Patients Infected with Pneumonia

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## Abstract

This study aimed for isolation and identification of *Klebsiella pneumoniae* from clinical samples and detection the prevalence of some extended spectrum β-lactamases (ESBLs) genes ($bla_{SHV}$, $bla_{CTX-M}$) in *K. pneumoniae* Samples collected were clinical respiratory tract infections. A total of 210 sputum specimens during the period from December 2018 to February. Growth on blood agar, MacConkey agar was identified by cultural, morphological and biochemical tests which revealed that: 62⁄210 (51.66%) gave positive growth for *K. pneumoniae.* All the 62 isolates of *K. pneumoniae* were screened for their antibiotic resistance against 21 antibiotics of different classes using disk diffusion method. The results showed that all the tested isolates were resistant to at least 6 antibiotics of which they were tested, hence the isolates were considered to be multidrug resistant. The results showed that all of the isolates were found to be resistant to β-lactam antibiotic (amoxicillin-clavulanate acid) Disk approximation test and not production of ESBLs by these isolates. All the 62 isolates were submitted to molecular detection of some ESBLsgenes ($bla_{SHV}$, $bla_{CTX-M}$) using PCR technique. The results showed that only 8(12.90%) isolates were carried $bla_{CTX-M}$ gene and 7(11.29%) isolates were carried $bla_{SHV}$ gene.

## Keywords

Klebsiella pneumonia; β-lactamases genes; Pneumonia

Hospital Acquired Pneumonia (HAP) is usually caused by more resistant bacteria, such as *"Klebsiella pneumoniae"*, *"Staphylococcus aureus"*, *"Pseudomonas aeruginosa"*, and *"Escherichia coli"*. Individuals with a serious impairment of their immune system become susceptible to pneumonia caused by so-called opportunistic germs, such as certain viruses, fungi, and bacterial related to tuberculosis (mycobacteria), which wouldn't ordinarily cause illness in normal individuals. To beat with its constant exposure to potentially infectious microbes, the lung depends on a hierarchy of defense mechanisms. [1].

*K. pneumoniae* is strains that can produce extended spectrum beta-lactamases enzymes (ESBLs) and become highly effective against different beta-lactam antimicrobials. On the other hand, extended spectrum β-lactamase (ESBL) producing bacteria are resistant to various antimicrobials classes leading difficult in treating diseases and called MDR bacteria [2].

Multidrug-resistant bacteria and ESBL producing

*K.pneumoniae* and other Gram-negative bacteria have worldwide distributions with high degree of prevalence in both hospitals and community [3]. Nearly about more than 390 different types of ESBLs have been recognized around the world, among which *blaSHV*, *blaTEM* and *blaCTX-M* were more prevalent [4].

## Materials and Methods

**Table 1.** Antibiotic Susceptibility Test (AST) Materials

| Antibiotic Class | Antibiotic Subclass | Antibiotic Name | Symbol | Content | Zone diameter (mm) (CLSI, 2018) | | | Origin |
|---|---|---|---|---|---|---|---|---|
| | | | | | S | I | R | |
| Penicillins | | | | | | | | Himedia, India |
| | Penicillins | Penicillin | P | 10 U | ≥19 | 13-18 | ≤ 12 | |
| β-lactams/ β-lactamase inhibitor combinations | | | | | | | | |
| | | Amoxicillin-clavulanate acid | AMC | 10 µg | ≥18 | 14-17 | ≤ 13 | |
| Cephems (parenteral) | | | | | | | | |
| | Cephalosporins | Cefotaxime | CTX | 30 µg | ≥26 | 23-25 | ≤22 | |
| | | Ceftazidime | CAZ | 30 µg | ≥21 | 18-20 | ≤17 | |
| | | Ceftriaxone | CRO | 30 µg | ≥23 | 20-22 | ≤19 | |
| | | Cefoperazone | CFP | 75 µg | ≥21 | 16-20 | ≤15 | |
| Aminoglycosides | | | | | | | | |
| | | Gentamicin | CN | 10µg | ≥15 | 13-14 | ≤12 | |
| | | Tobramycin | TM | 10µg | ≥15 | 13-14 | ≤ 12 | |
| Fluoroquinolones | | | | | | | | |
| | | Ciprofloxacin | CIP | 5 µg | ≥21 | 16-20 | ≤ 15 | |
| | | Levofloxacin | LEV | 5 µg | ≥17 | 14-16 | ≤ 13 | |
| | | Norfloxacin | NOR | 10 µg | ≥17 | 13-16 | ≤ 12 | |
| Floate pathway inhibitors | | | | | | | | |
| | | Trimethoprim | TMP | 5µg | ≥16 | 11-15 | ≤ 10 | |
| Cephems (oral) cephalosporins | | | | | | | | |
| | | Cefpodoxime | CPD | 10 µg | ≥21 | 18-20 | ≤ 17 | |
| Monobactams | | | | | | | | |
| | | Aztreonam | ATM | 30µg | ≥21 | 18-20 | ≤ 17 | |
| | | Imipenem | IPM | 10 µg | ≥23 | 20-22 | ≤ 19 | |
| Tetracyclines | | | | | | | | |
| | | Tetracycline | TE | 30 µg | ≥15 | 12-14 | ≤ 11 | |
| Nitrofurans | | | | | | | | |
| | | Nitrofurantoin | NIT | 300 µg | ≥17 | 15-16 | ≤ 14 | |
| Phenicols | | | | | | | | |
| | | Chloramphenicol | C | 30 µg | ≥18 | 13-17 | ≤ 12 | |
| Quinolones | | | | | | | | |
| | | Nalidixic acid | NA | 30 µg | ≥19 | 14-18 | ≤ 13 | |
| Another Antibotic | | | | | | | | |
| | | Cefotaxime-Clavulantea | CEC | 30µg | ≥26 | 23-25 | ≤22 | |

S, sensitive; I, intermediate; R, resistance; µg, microgram; mm, millimeter.

**Table 2.** Primers for Detection Genes of ESBL producing K. pneumonia

| Target Genes | Primer Sequence (5′-3′) | | Size (bp) | References |
|---|---|---|---|---|
| $bla_{SHV}$ | F | 5′- ATTTGTCGCTTCTTTACTCGC -3′ | 1018 | (Jemima and Verghese, 2008) |
| | R | 5′- TTTATGGCGTTACCTTTGACC -3′ | | |
| $bla_{CTX-M}$ | F | 5′- ATGTGCAGYACCAGTAARGT -3′ | 544 | |

| | | | | |
|---|---|---|---|---|
| | R | 5′- TGGGTRAARTARGTSACCAGA -3′ | | |

*bla*$_{SHV}$ β-lactamase is sulfhydryl variable; *bla*$_{CTX-M:}$ β-lactamase is cefotaxime hydrolyzing capabilities; bp: base pair; F: forward; R: reverse.

**Table 4.** Programs of multiplex PCR for blaSHV and blaCTX-M genes in the ESBL producing K. pneumonia

| Multiplex Genes | Steps | Reaction Conditions | Cycle no. | References |
|---|---|---|---|---|
| *bla*$_{SHV}$ and *bla*$_{CTX-M}$ | Initial denaturation | 94 °C for 5 min. | 32 | (Jemima and Verghese, 2008) |
| | Denaturation | 94 °C for 45 sec. | | |
| | Annealing | 50 °C for 40 sec. | | |
| | Extension | 72 °C for 60 sec. | | |
| | Final extension | 72 °C for 10 min. | | |

*bla*$_{SHV}$: β-lactamase is sulfhydryl variable; *bla*$_{CTX-M}$: β-lactamase is cefotaxime hydrolyzing capabilities; °C: Celsius; sec: second; min: minute; no: number

## Patients and Clinical Specimens

A total of 285 individuals in different sex and age groups cases (148 males and 137 females), including 210 clinical samples of patients which from respiratory tract and 75 healthy as a control group, between December 2018 and February 2019 in Diwaniya Teaching Hospital in Diwaniya Province clinical Samples were collected by using swabs from sputum and nares. The sterile cotton swabs were immersed in BHI agar tubes and transferred to the laboratory [5]. The Isolates were identified depending on the morphological features on culture medium and biochemical tests according to the classification of [6]. The smears from all isolates stained by the Gram's technique and examined under the oil immersion lens of the light microscope X 100, to detect the staining reaction, sizes, shapes, and arrangement of the cells [7]. All samples were inoculated on to MCA and BA and incubated at 35˚C for 24 hours. Colonies were purified by sub-culturing for further identification. Color, shape, size, edge, type of hemolysis and lactose fermenter were observed [7]. The biochemical tests were (lactose fermented ,growth on MacConkey agar, catalase , oxidase blood hemolysis ) tests , While the antibiotics susceptibility testing occur by: Disc Diffusion (DD) Method :This is the most thoroughly described D.D method on MHA medium for which interpretive standards have been developed and supported by CLSI data [8].

The β-Lactamase production occur by cloverleaf test: Plate of MHA was inoculated with *E. coli* ATCC 25922. A penicillin disc (10 U) was placed in the center of the plate and four test isolates were streaked radially outward from the disc to produce growth about 0.25 cm wide. The plate incubated at 37˚C for 18 hours and examined, if the isolate produced β-lactamase, giving rise to a cloverleaf pattern [9]. And all test isolates were streaked on ESBL CHROM agar. Plates were incubated aerobically at 35˚C for 48 hours. ESBL producing *K. pneumonia* colonies were appeared blue to greenish .The genomic DNA extraction for molecular study according to Promega kit. Optimization of PCR was accomplished after several trials, so the following mixture was according to information of iNtRON Biotechnology PCR protocol.

## Results and Discussion

The result of Gram stain of sputum specimens were 90 (42.85%) specimens Gram positive and 120 (57.14%) specimens Gram negative (Figure 1).

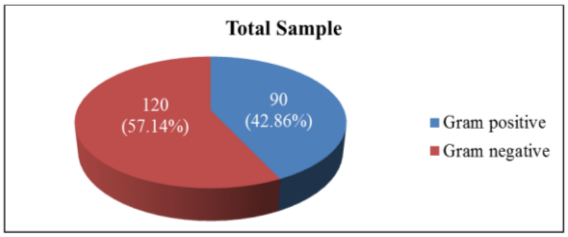


**Figure 1:** Results of Gram stained smears of 210 sputum specimens were collated from patients with pneumonia.

From 120 sputum specimens that showing negative result for Gram stain, 62 (51.66%) specimens have been appeared positive result for *K. pneumonia* when cultured on cultural media , while 54 (45%) of specimens were negative results and 4 (3.33%) of specimens weren't appeared growth in cultural media. The results revealed that *K. pneumoniae* causing different infections, it was identified in 62 (51.66%) isolates from 120 sputum specimens that showing negative result for Gram stain. This result is in agreement with many studies established

by [10] which have concluded that *K. pneumoniae* was recorded in 54 (54.4%) isolates.

However the members of *K. pneumoniae* causing infectious diseases were vary according to the geographic area, period of isolates collection and different bacterial diagnostic methods. [11] revealed that *K. pneumoniae* accounted 44% and *K. oxytoca* comprised 16% from the total number of Gram-negative bacilli. *K. pneumoniae* in study of [12] recovered from 50% of all Enterobacteriaceae isolates.as shown study (Figure 2).

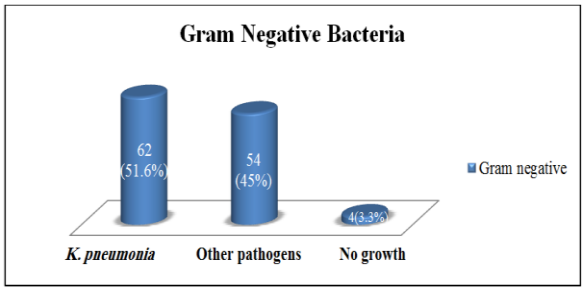


**Figure .2:** Results of bacterial isolation from 120 sputum specimens on cultural media.

Sixty tow isolates were belonging to *K.* pneumonia positively from respiratory tract infections, 37 (59.7%) of them were males and 25 (40.3%) female (Figure 3). [13] revealed that *K. pneumoniae* accounted 44% of a total infections in human body and This is higher from than obtained in the present study. Besides, this result did not agreed with other study which found that the percentage of *K. pneumoniae* recovered from clinical specimens ranged between 8-9% [14].

This result is confirmed by the finding obtained by [15] who found that immunocomprised patients are colonized by *K. pneumonia* and agreed with the finding obtained by [16] who found that *K. pneumonia* were responsible for nosocomial infection in adult in Georgia hospital.

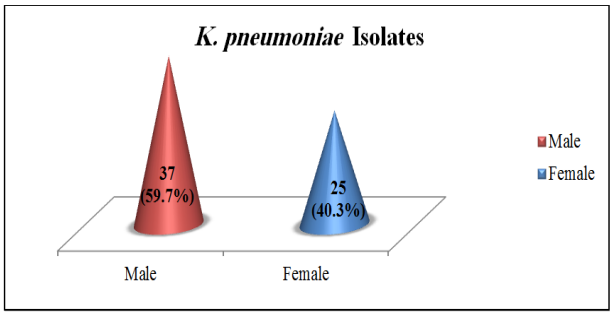


**Figure .3:** Frequency of K. pneumoniae in clinical samples collected from both sex.

Colonies of *K. pneumoniae* were observed, rounded, smooth, convex and Gamma hemolytic when cultured and streaked on BA [17]. While the colonies on MCA which appeared typically large, mucoid, with pink to red pigment, usually diffusing into the surrounding agar, indicating fermentation of lactose [18] (Figure 4). The results of microscopic examination showed that the cells of bacterial isolates appeared Gram negative rod, non-motile and non-spore forming bacteria [19] (Figure.5) .

Gram-negative isolates that grown on MCA undergo biochemical tests in order to distinguish *K. pneumoniae* isolates from other members of related lactose fermented bacteria, all biochemical tests have been carried out according to [6] (Table 5). It were positive results for catalase test, While negative results for oxidase test, [20] (Figure 6).

Nosocomial infections due to Klebsiella species are a major cause of morbidity and mortality among several patient populations. Of these groups, burn patients are particularly susceptible to life-threatening infections. *K. pneumonia* was recorded second agent after *Pseudomonas aeruginosa* that caused bacteremia in respiratory tract infection patients, Klebsiella infections are encountered far more often now than in the past. This is probably due to the bacterium's antibiotic resistance properties [1].

According to the site of infection, the results of the present study are similar with many studies like [15] who found that *K. pneumoniae* was the most causative agent that cause urinary tract infection , respiratory tract infection and blood infection with percentage range 10% and 15%, respectively, and this fact was in accordance to [21] who they are reported that *K. pneumoniae* is associated with aspiration pneumonia , thus the 2nd common site of samples collection was the endotracheal secretion and burns infections.

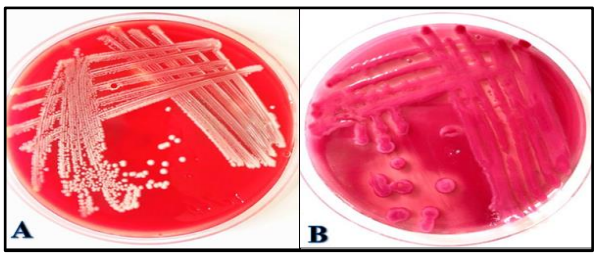


**Figure .4:** Cultural and morphological characteristics for detection of K. pneumoniae. (A): Blood agar were colonies rounded, convex and Gamma hemolytic (B): MacConkey agar were colonies large, mucoid, with pink to red pigment and fermentation of lactose.

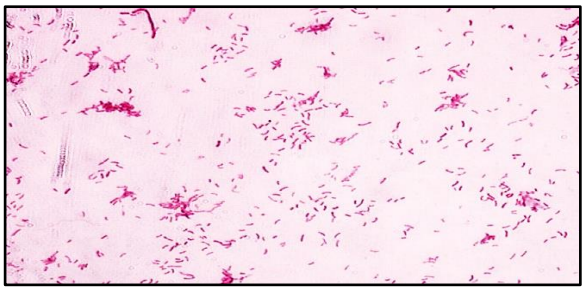

**Figure .5:** Microscopically diagnosis of Gram stain rod and non-spore of K. pneumoniae.

**Table 5:** biochemical tests of K. pneumoniae isolates from clinical samples

| Biochemical Test | Result |
|---|---|
| Catalase | +ve |
| Citrate | +ve |
| Gamma hemolytic | Non-hemolytic |
| Gram stain | -ve |
| Indole | -ve |
| Lactose | +ve |
| Lipase | -ve |
| Methyl Red | -ve |
| Oxidase | -ve |
| Protease | -ve |
| Sucrose | +ve |
| Spore | -ve |
| Vogas-Proskauer | +ve |

+ve: Positive result, - ve: Negative result.

Susceptibility of *K. pneumoniae* isolates under study for common antibiotics was studied to determine the pattern of resistance to various antibiotics depending on disk diffusion method. The antibiotics represented by penicillins, β-lactams, cephems (parenteral), aminoglycosides, tetracyclines, quinolones, fluoroquinolones, monobactams, floate pathway inhibitors, phenicols, cephems (oral) cephalosporins and Nitrofurans.

The diameters of inhibition zones around the antibiotic disks were measured and compared with the standard manual of [22]. The results have clarified that the *K. pneumoniae* isolates showed high resistance to penicillins, all of isolates 62 (100%) were resistant to penicillin. Resistance to amoxicillin clavulanate acid was found in 59 (88.3%) isolates while 3 (11.6%) isolates sensitive in present study which is close to the finding of [23] which were 87.5% of *K. pneumoniae* isolates appeared resistance to amoxicillin clavulanate acid.

This result agreed with other studies [24]. They found that all strains of *K. pneumoniae* are resistant to pencillin and amoxicillin clavulanate acid. The resistance to cephalosporins were represented by 54 (87%) resistant to both cefpodoxime and ceftriaxone, while 59 (95.3%), 51 (82%) and 45 (72%) isolates were resistant to cefotaxime, cefoperazone and ceftazidime respectively.

The high resistance was related to different mechanisms which mediate antibiotic resistance in *K. pneumoniae* that were recorded by several studies such as [25] who recorded that the resistance of *K. pneumoniae* to β-lactams range from 63.3% to 70% which is less than the result of the present study, this may be due to the exposure of *K. pneumoniae* to antibiotic resulting from random use of antibiotic during infectious diseases inducing *K. pneumoniae* to persist under these drastic conditions and overcome antibiotic treatment [1] demonstrate that *K. pneumoniae* can resist cephalosporins, penicillins and other β-lactams via alternating the permeability of plasma membrane preventing antibiotics from entry to the bacterial cell.

Revealed that *K. pneumoniae* produce several extended spectrum β-laclamase enzymes which inhibit the action of β-lactam antibiotics [26], conferring bacteria the ability to persist under β-lactams treatment, as recorded by [27], the latter pointed out that 69%, 74% and 79% of *K. pneumoniae* isolates were resistant to ceftazidime, cefotaxime and ceftriaxone respectively. American proficiency institute (2008) recorded that 94% of *K. pneumoniae* isolates were resistant to penciline, but are highly susceptible to ceftazidime (76.4%) but less so to ceftriaxone (35.2%).

The results revealed that all of *K. pneumoniae* isolates were sensitive to monobactams (imipenem) 56 (90.3%) (Figure 2), this result goes in agreement with the result of [27]; therefore, imipenem is the drug of choice to treat infections caused by extended spectrum β-lactamases-producing *K. pneumoniae*.

The results revealeded that impenem was the best choiced antibiotic for treatment of *Klebsiella* infection , since the effect of impenem was appear in all bacterial isolates (100%) .This result agreement with study of [15] they found that all *K. pneumonia* were sensitive to impeneme 100%. Aztreonam (which is a member of monobactams) was active against 20 (32.3%) isolates therefore 38 (61%) of *K. pneumoniae* isolates were resistant, this results was same results recorded by [28] (63.6%) and the results of American Proficiency Institute, (2008) were (75.4%).

The resistance to aminoglycosides were recorded in 30(48%) and 23(37%) isolates to tobramycin and gentamycin respectively, versus 10(16.2%) and 4(6%) isolates were intermediate resistant to antibiotics mentioned as above respectively. These results illustrate that aminoglycosides are more active against *K. pneumoniae.* than β-lactams, our result of gentamycin resistance disagreement with the result recorded by [27] which were (92.6%).

The resistance pattern of *K. pneumoniae* isolates to tetracycline, ciprofloxacin, norfloxacin, levofloxacin, nalidixic acid and nitrofurantoin were represented by 30(48%), 34(54%), 26(41%), 24(38%), 40(64%) and 32(51%) isolates respectively, while 9(14%) and 7(16.2%), 2(3%) of isolates gave intermediate resistance to tetracycline, ciprofloxacin, levofloxacin.

Recorded that 68.8% of *K.pneumoniae* were susceptible to ciprofloxacin [29]. The results of the present study have revealed that 56 (90%) of *K.* pneumoniae isolates was resistant to trimethoprim which is a member of sulfonamides; this finding is close to the result reported by [27] who found that 79% of *K.pneumoniae* isolates were resistant to trimethoprim.

The present study recorded that 38 (61%) and 4 (6%) of *K. pneumoniae* isolates were resistant and intermediate resistant to chloramphenicole respectively this results is lower than those recorded by [30], which were 40.6%.

**Table 6:** Antibiotics sensitivity test for 32 clinical isolate of K. pneumoniae.

| Antimicrobial agent | No. (%) of isolates | | |
|---|---|---|---|
| | R | I | S |
| Amoxicillin-clavulanate acid | 59(95.16) | 0(00) | 3(4.83) |
| Aztreonam | 38(61.3) | 4(6.5) | 20(32.3) |
| Cefoperazone | 38(61.3) | 8(13) | 16(25.8) |
| Ceftazidime | 45(72.58) | 0(00) | 17(27.41) |
| Cefotaxime | 59(95.16) | 0(00) | 3 (4.83) |
| Cefpodoxime | 54(87) | 0(00) | 8(13) |
| Ceftriaxone | 54(87) | 0(00) | 8 (13) |
| Ciprofloxacin | 34(54.83) | 0(00) | 28(45.16) |
| Chloramphenicol | 38(61.29) | 4 (6.45) | 20(32.25) |
| Gentamicin | 23(37.09) | 4(6.45) | 35(56.45) |
| Imipenem | 6(9.6) | 0(00) | 56(90.3) |
| Levofloxacin | 24(38.70) | 2(3.22) | 36(58.06) |
| Nalidixic acid | 40(64.51) | 0(00) | 22(35.48) |
| Nitrofurantoin | 32 (51.61) | 0(00) | 30 (48.38) |
| Norfloxacin | 26(41.93) | 0(00) | 36(58.06) |
| Penicillin | 62(100) | 0(00) | 0(00) |
| Tobramycin | 48(77.4) | 0(00) | 14(22.6) |
| Tetracycline | 30(48.38) | 9(14.51) | 23(37.09) |
| Trimethoprim | 56(90.3) | 0(00) | 6(9.6) |

The results of the current study showed that all bacterial isolates 62 (100%) were able to grow in the presence of penicillin by cloverleaf test (Figure 5). previous methods was used for detection penicillinase production. This result is considered that these isolates are resistant to β-lactam antibiotics according to phenotypic detection [31] (Table 4.5) β-lactam antibiotics extensive use in communities and hospitals has generated main problems leading to increased mortality, morbidity and health care cost [32].

In cloverleaf method, if the test isolate produced β-lactamase, the growth coincide thus giving rise to a cloverleaf pattern. In the current study, cloverleaf technique did not get any false results and this agreed with the fact that this test depend on the β-lactamase produced by one organism allowing an indicator isolate to grow, there is nil chance of getting false positive results [31]. Additionally, this technique found to be the easy, cost effective, and reliable method for detection of β-lactamase. This result was confirmed with [31].

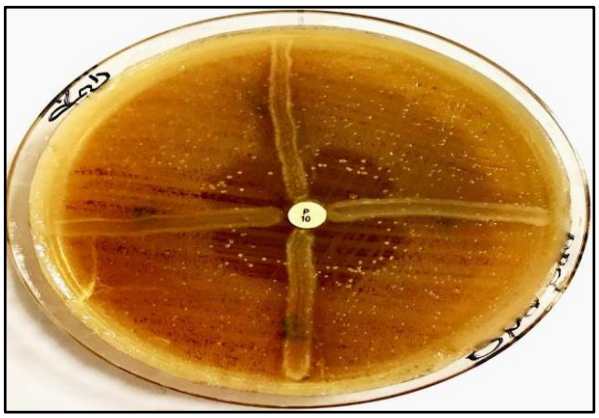

**Figure (6):** β-lactamases production detected by cloverleaf method: Positive result giving rise to a cloverleaf pattern around [penicillin (P10U)] disc by isolates NA-12, 16, 32, 45).

All 62 β-lactam resistant *K. pneumoiae* isolates were screened for ESBL production according to the Clinical and Laboratory Standard Institute criteria 2018. ESBL production among these isolates were detected phenotypically by CHROM agar confirmatory test results of the present study confirmed the presence of an ESBL in 20 (32%) of β-lactam resistant isolates. All these isolates showed overnight growth with blue colonies on the ESBL supplemented CHROM agar orientation medium while ESBL producers (Figure 6).

Also the results of the present study were similar with [33] who is showed that *K. pneumoniae* was the most dominant ESBL producing bacteria with percentage 65.8%. [26] suggested that repeated use of

third generation of cephalosporins antibiotics against bacterial infections are became the main risk factors, because these bacteria can take other ways for resistance to these antibiotics by producing enzymes of ESBLs.

But the results of the current study are non in agreement with [20] who is found that 6 out of 28 isolates (30.4%) were ESBL-producing *K. pneumoniae*. Although earlier reports have suggested that ESBLs positive strains are generally resistant to other antibiotics, but the high percentage of multi drug resistance strains indicated an relationship with ESBL positive strains (97%) [34].

Reported that (60.4%) of *K. pneumoniae* isolates were produced ESBL amase which are closely related to the present study [35]. The rate of revealing of ESBLs producing *K. pneumoniae* isolated from clinical samples differs from each other, screening for the presence of ESBL among *K. pneumoniae* carried out in study of [23] who is reported that the highest rate of ESBL production was found in *K. pneumoniae*. with percentage (51.2%) followed by *E. coli* (40.2%), *E. aerogenes* (33.4%) and *P. aeroginosa* (27.9%). During few years ago, the occurrence of ESBL producing strains among clinical isolates of *K. pneumoniae* have been on steady increase, therefore accounts about more than 50% of all nosocomial infections were ESBL producing *K.* pneumoniae isolates [36].

The current study suggests that MDR and ESBL *K. pneumoniae* isolates are a horizontal dissemination of these determinants amongst clinical isolates of *K. pneumoniae*. These bacterial strains co-carrying diverse and numerous multiple resistance determinants may impose limitations in the therapeutic options available for the treatment of infections. The results is similar with [33] who reported that (85%) and (65.7%) of *K. pneumoniae* isolates were positive for ESBLs. Also [37] reported that (58%) of *K. pneumoniae* isolateswere ESBLs.

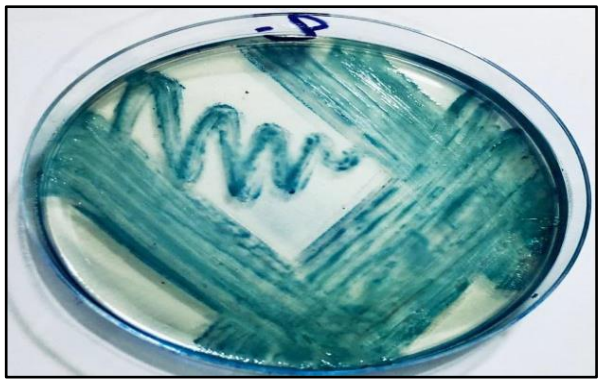

**Figure 7:** ESBL production by K. pneumoniae isolate exhibit deep blue colonies on the ESBL supplemented CHROM agar medium

β-lactam antibiotics are the broad class of anti-bacterial drugs classified according to their chemical structures, bacteria especially Gram negative are able to resist the killing action of these drugs through their ability to produce β-lactamase enzymes such as *CTX-M* and *SHV* [38]. Repeating using of β-lactam antibiotics against bacterial infections have induced mutation in these bacteria and lead to production of new β-lactamases enzymes, that lead to expanding of bacterial strains even against the newly synthesized β-lactam antibiotics, these newly β-lactamases enzymes are called extended-spectrum β-lactamases [39].

Polymerase chain reaction technique has been used to amplify the genes encoding the *SHV and CTX-M* β-lactamases from genomic DNA of *K. pneumonia* isolates. The results of the current study demonstrated that out of total 20 isolates, 8 isolate (93.54%) were positive for $bla_{CTX\text{-}M}$ gene and 7 isolates (87.09%) were positive for $bla_{SHV}$ gene, respectively (Figures 8 and9). Many types of bacteria especially *K. pneumoniae* and *E. coli* still the major ESBLs producing microorganisms isolated worldwide. Prevalence of ESBL varies from a researcher to another. Many studies from Asia have reported ESBL production varying from 10 to more than 90% [40].

In current study, production of ESBL was examined by DDS and confirmatory tests, the DDS test was carried out according to the size of the inhibition zone around ceftazidime, cefotaxime and ceftriaxone. The results demonstrated that out of 62 isolates there were 20 isolates (72.58 %) were ESBLs producers (Figure 4-4). This results are in agreement with [4] who they are indicated that about 70% of *K. pneumoniae* were ESBL producing bacteria.

Proved that the ESBL phenotype in *K. pneumoniae*

strains that isolated from different hospital environments were identified in (97%) of the strains positive for resistance to 3rd generation cephalosporins [41]. [4] reported that the prevalence of *bla*$_{CTX-M}$ gene in total isolates of *K. pneumoniae* were (20%) and the prevalence of *bla*$_{SHV}$ gene in total isolates of *K. pneumoniae* were (8.4%), on the other hand the total isolates having both *bla*$_{CTX-M}$ and *bla*$_{SHV}$ genes were (67.3%).

Many studies similar to this study such as the manuscript of [42] that documented that the prevalence of *bla*$_{CTX-M}$ gene in total isolates of *K. pneumoniae* were 9 isolates only (34.61%) and the prevalence of *bla*$_{SHV}$gene in total isolates of *K. pneumoniae* were 12 isolates with percentage (46.15%). In other study by [35] who proved that only 3 out of 30 isolates (10%) of *K. pneumoniae* were positive for *bla*$_{SHV}$ gene. The results of the current study are in agreement with [42] who reported that the prevalence of *bla*$_{SHV}$ gene in *K. pneumoniae* isolates were (88%), on the other hand , in the same study , prevalence of *bla*$_{CTX-M}$ gene was (34.61%) of total *K. pneumoniae* isolates.

The epidemiological investigation of ESBL positive strains these strains remains important because of infections caused by these strains are related to high mortality and morbidity rates due to resistance to other antibiotics [43].

In Asia prevalence of ESBL producers among *K. pneumoniae* a commonest nosocomial associated bacteria is reported to be more than 50% [44]. Study of the development and variety of drugs resistance genes in normal populations is important for knowing of the origin of resistance and of the evolution of like, β-lactamase catalytic activity. Indication that the genes resistance diversity was proved by firstly studies relating β-lactamases and penicillin binding proteins [45]. The ESBL are usually used practically for the treatment of infections caused by Gram negative bacteria, but the rise of ESBL producing organisms has modeled a severe threat for their current use [46].

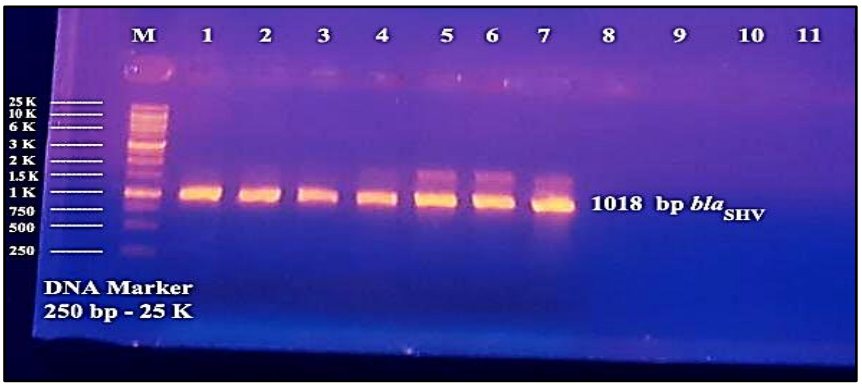


**Figure 8:** multiplex PCR amplified products from extracted total DNA of K. pneumoniae isolates isolated from different clinical specimens. Lane: (1 to 54 isolates) amplified with SHV gene, show positive results at 1018 bp . The electrophoresis was performed at 80 volt for 95 Minutes. (L): DNA molecular size marker (100bp ladder , 100 to 3000 bp).

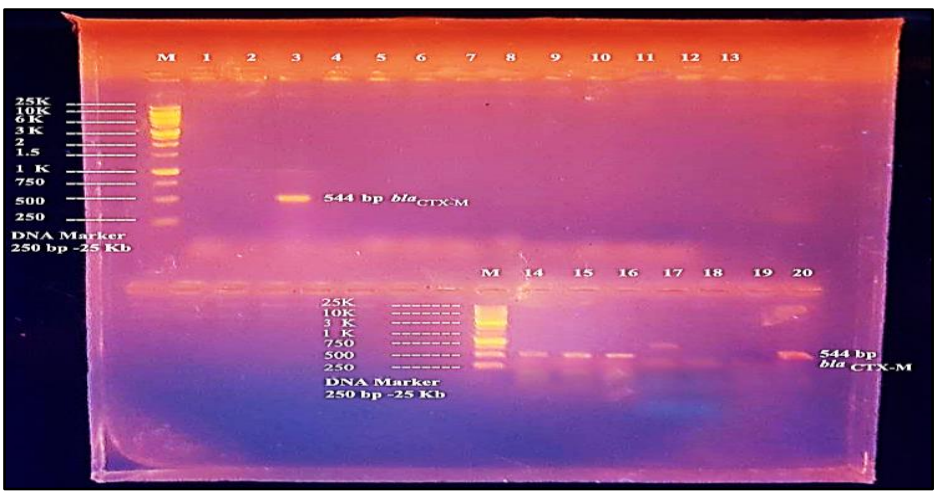

**Figure 9:** Ethidium bromide-stained agarose gel electrophoresis of multiplex PCR for K. pneumoniae isolates isolated from different Lane: 1-7 isolates amplified with CTX-M gene, show positive results at single band at 544 bp . The electrophoresis was performed at 80 volt for 95 Minutes. (L): DNA molecular size marker (100bp ladder , 100 to 3000 bp)

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