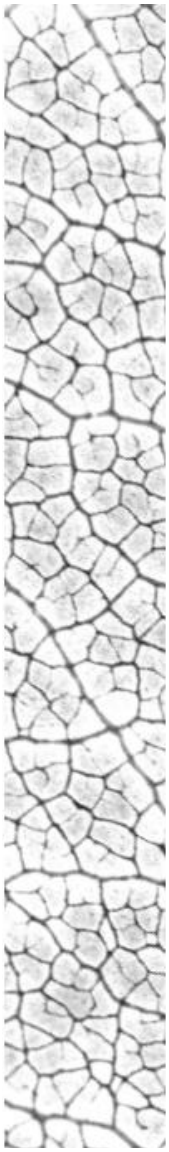

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Molecular detection of β-lactamase production among klebsiella pneumoniae isolated from different clinical cases

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Abstract
Many bacteria around the world produce the extended-spectrum β-lactamase (ESBL), especially *Klebsiella pneumonia*e, which are resistant to many beta lactam antibiotics by coding genes of enzymes that in turn give *K.pnemoniae* antibiotic resistance, as well as a role in the development of infection. The isolates of *K.pneumoniae* were identified based on phenotypic and laboratory methods, as well as screening of β-lactamase by phenotypic confirmatory test (PCT) where most isolates were positive at a rate (62.06%). *K.penumoniae* isolates resistance was also tested from a total of (110) isolates from various clinical sources: urine (72) and *K.pneumonia* isolates were diagnosed about 16 (22.22%, 16/72) , sputum (25) were diagnosed about 8 (32%, 8/25) and burns (13) about 5 (38.46%, 5/13) different resistance to 10 beta-lactam antibiotics including piperacilin (100%), ticarcillin (82.75%), aztreonam (79.31%), ceftazidime (58.62%), ceftriaxone (48.27%), augmentin (41.37%), ceftaxime, meropenem (37.93%), cefepime and imipenem (34.48%). In this study molecular diagnostics for *16S* RNA gene screening, as well as four genes for beta-lactamase were investigated. The results showed that high genes of these enzymes were βla_{TEM}, βla_{SHV} (80%) and βla_{CTX-M}, βla_{AmpC} (100%). It results in a positive relationship between the existence of genes and the of antibiotic resistance in isolates.

Keywords: klebsiella pneumonia, βlatem, βlashv, βlactx-m and βlaampc genes, esbls, antibiotic resistant

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INTRODUCTION

Expanded range β-lactamase (ESBL) creating Escherichia coli and Klebsiella pneumoniae have spread quickly worldwide and represent a genuine danger in medicinal services related contaminations (Kiratisin et al. 2008). *Klebsiella pneumoniae* is one of the most significant pathogenic microscopic organisms, it is (- ve), bacilli, nonmotile and causative specialist of numerous maladies, for example, pneumonia, urinary tract contaminations (UTIs) and intra-stomach diseases for patients with extreme basic illnesses among medical clinic immunocompromised (Rahamathulla et al. 2016). Antimicrobial resistance is an increasing problem in many bacterial pathogens and is of particular concern for nosocomial infections in hospitals (Monnet et al. 1998). In recent years, the misuse and increased use of antibiotics has resulted in a serious global outcome (Nordmann et al. 2011). Difficult treatment of these infections which cause pathogen-producing ESBL to remain in the environment and patients for a long period of time and to easily spread within and between hospitals within a few years of the release of β-lactams, gram-negative bacilli, in particular *K.pneumoniae*, that detected harbor mutated versions of the potent TEM and SHV enzymes and others (third generation cephalosporins and aztreonam) (Emery and Weymouth 1997, Wassef et al. 2014, Paterson et al. 2004). Another issue has risen in enteric bacteria: plasmid-intervened AmpC proteins, They are gotten from chromosomal AmpC genes of gram-negative life forms, AmpC enzymes are commonly impervious to broad-spectrum penicillins, broadened range cephalosporins, monobactam, and cephamycins however are helpless to cefepime, cefpirome, and carbapenems (Philippon and Jacoby 2002). The objective of this research was to investigate the mechanism of beta-lactam resistance of *K.pneumoniae* isolates from various clinical infections and molecular diagnosis of β-lactamase genes such as βla_{TEM}, βla_{CTX-M}, βla_{SHV} and βla_{AmpC}.

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Table 1. The ESBL gene primer sequences in the current study

Primer	Sequence (5'to 3')	Sequence	Size (bp)	References
16S rRNA	F-	CCTGGACAAAGACTGACGCT	584	Designed by the researcher using plus - 3
	R-	AGTTGCAGACTCCAATCCGG		
SHV	F-	CCGCCATTACCATGAGCGAT	410	
	R-	AATCACCACAATGCGCTCTG		
TEM	F-	GGTGCACGAGTGGGTTACAT	531	
	R-	TGCAACTTTATCCGCCTCCA		
AmpC	F-	AAACGACGCTCTGCACCTTA	670	
	R-	TGTACTGCCTTACCTTCGCG		
CTX-M	F-	AGCGATAACGTGGCGATGAA	247	
	R-	TCATCCATGTCACCAGCTGC		

MATERIAL AND METHODS

This study was conducted from June (2018) to January (2019) in the general teaching hospital of AL-Diwaniya Town, including blood, sputum and burns, numerous medical specimens are collected from different clinical samples. The selection process was carried out according to the (Friedrich et al. 2005).

Distinguishing of Bacterial Isolates

All isolates are described by traditional microscopic examination (Gram's stain), morphological colony characteristics on macConkey agar and normal bacteriological tests (Podschun and Ullmann 1998).

Antibiotic Susceptibility Test

Antibiotic susceptibility testing of all isolates was determined in this study using the CLSI guideline disk diffusion method (Clinical and Laboratory Standards Institute 2012, Testing for antimicrobial susceptibility on Mueller-Hinton ager (oxoid, UK) was formed. The following antibiotic disks have been used: Augmentin (AUG:30 Mg), Ticarcillin (TIC:75 Mg), Meropenem (MEM:10 Mg), Ceftriaxone (CRO:30 Mg), Aztreonam (AZ: 30 Mg), Cefepime (FEP: 30 Mg) piperacilin (PIP: 100 Mg), Imipenem (IMP:10 Mg), Cefotaxime (CTX: 30 Mg), Ceftazidime (CAZ: 30 Mg). It was collected from antibiotic disks (Bioanlyse, Turkyi). Control organism was bacteria *E.coli* ATCC 25922 strain.

Production of ESBL

The ability of bacteria to produce β-lactamase was investigated using phynotypic confirmatory test (PCT): was placed Augmentin disc (30mg) in the focal point of Mueller Hinton agar and around it on three sides of the plate, including ceftriaxone (30mg), cefotaxime (30mg) and ceftazidime (30mg). The plate was then incubated in the incubator at 37C° for 24h.If the inhibition zone increases towards augment in disc, the result considered positive for beta-lactamase production (Sarojamma and Ramakrishna 2011).

DNA Extraction and PCR

K.pneumoniae's complete genomic DNA was extracted according to the manufacturer's instructions using the DNA extraction and purification kit (Bioneer company, Korea) According to guidance from the manufacturer. DNA preparation was then analyzed in 1,5 percent agarose gel by electrophoresis. PCR was used in this study to amplify the whole gene sequences tested. The different primers used to amplify these genes (Bioneer company, Korea) were shown in (**Table 1**). PCR mixtures included: Top DNA polymerase 1U, dNTP (dATP, dCTP, dGTP, dTTP), Tris-HCL (pH 9.0) 10mM, KCL 30mM, MgCL2 1.5mM each. The thermocycling conditions of the polymerase chain reaction (PCR) have been shown in (**Table 2**).

Table 2. PCR thermocycling conditions

PCR step	Temp. (C°)	Time	Repeat
Initial Denaturation	95	5 min.	1
Denaturation	95	30 sec.	30 cycle
Annealing	58	30 sec.	
Extension	72	1 min.	
Final extension	72	5 min.	1
Hold	4	Forever	-

Table 3. Numbers and percentage of K.pneumoniae by total isolates of bacteria and infection sites

Site of infection	Bacterial isolates	*K.pneumoniae*	Percentage (%)
Urine	72	16	22.22
Sputum	25	8	32
Burns	13	5	38.46
Total	110	29	26.36

Table 4. Percentage of Antibiotics resistance and sensitivity of *K.pneumoniae*

Antibiotics	Resistince isolates		Sensitie isolates	
	No.	(%)	No.	(%)
Augmentin	12	41.37	17	58.62
Ticarcillin	24	82.75	5	17.24
Meropenem	11	37.93	18	62.06
Ceftriaxone	14	48.27	15	51.72
Aztreonam	23	79.31	6	20.68
Cefepime	10	34.48	19	65.62
Piperacilin	29	100	0	0
Imipenem	10	34.48	19	65.51
Cefotaxime	11	37.93	18	62.06
Ceftazidime	17	58.62	12	41.37

RESULTS

Total 110 specimens were collected from different clinical sources, there were 29 specimens (26.36%) it has been diagnosed as *K. pneumoniae* , they were 16 isolate (22.22%) from urine, 8 isolates (32%) from sputum and 5 isolates (38.46%) from burns (**Table 3**).

All isolates of *K.pneumoniae* showed microbiological resistance in (**Table 4**), the highest rate of resistance was to piperacillin (100%) and the lowest to imipenem and cefepime were (34.48%).

The purified PCR products for DNA extraction of *K.neumoniae* isolates by using *16S rRNA* gene , were detected (100% , n=29) (**Fig. 1**).

The results showed that 10 isolates resistant beta-lactam antibiotics contained ESBL genes (Bla_{TEM} , Bla_{CTX-M}, Bla_{SHV} and Bla_{AmpC}) (**Table 5**) were detected Bla_{TEM} gene about (80%) (**Fig. 2**), Bla_{CTX-M} gene about (100%) (**Fig. 3**), Bla_{SHV} gene about (80%) (**Fig. 4**) and Bla_{AmpC} gene about (100%) (**Fig. 5**).

Table 5. The numbers and percentages ESBL genes for 10 *K.pneumoniae* isolates

ESBL genes	No. *K.*pneumoniae isolates	(%)
TEM	8	80
CTX-M	10	100
SHV	8	80
AmpC	10	100

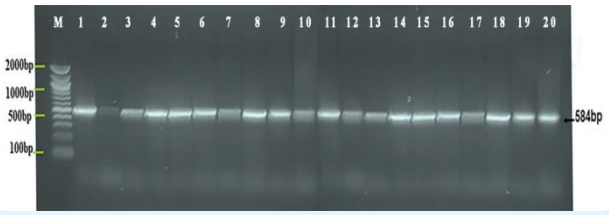

Fig. 1. Agarose gel 1.5 percent electrophoresis, the *16SrRNA* gene PCR result isolates of *Klebsiella pneumoniae*. Where M: Marker (100-2000bp), lane (1-20) positive PCR (584bp) PCR product size amplification

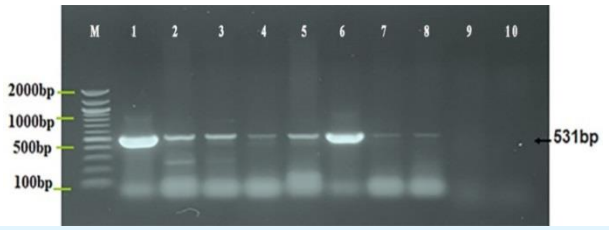

Fig. 2. Agarose gel 1.5 percent electrophoresis, the *βla*$_{TEM}$ gene PCR result isolates of *Klebsiella pneumoniae*. Where M: Marker (100-2000bp), lane (1-8) positive PCR (531bp) PCR product size amplification

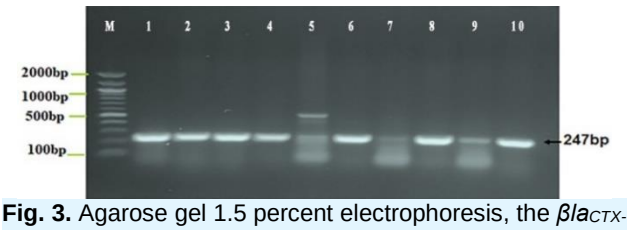

Fig. 3. Agarose gel 1.5 percent electrophoresis, the *βla*$_{CTX-M}$ gene PCR result isolates of *Klebsiella pneumoniae*. Where M: Marker (100-2000bp), lane (1-10) positive PCR (247bp) PCR product size amplification

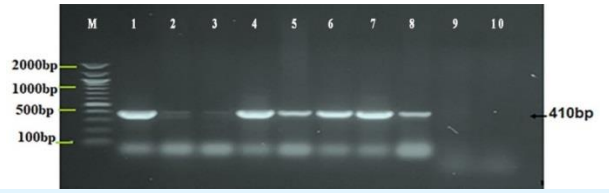

Fig. 4. Agarose gel 1.5 percent electrophoresis, the *βla*$_{SHV}$ gene PCR result isolates of *Klebsiella pneumoniae*. Where M: Marker (100-2000bp), lane (1-8) positive PCR (410bp) PCR product size amplification

DISCUSSION

K. pneumoniae is a significant nosocomial pathogen that can cause morbidity and mortality in severs. Many

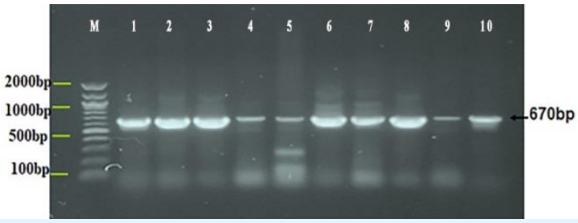

Fig. 5. Agarose gel 1.5 percent electrophoresis, the *βla*$_{AMPC}$ gene PCR result isolates of *Klebsiella pneumoniae*. Where M: Marker (100-2000bp), lane (1-10) positive PCR (670bp) PCR product size amplification

reports on outbreaks in various health care settings have been published worldwide. *K.pneumoniae* is highly resistant to penicillins, including semi-synthetic penicillins of broad spectrum. Outbreaks of infection caused by extended spectrum beta lactamase producing by *K.pneumoniae* have been widely reported worldwide in recent years following extensive use of the expanded spectrum of cephalosporines (Branger et al. 1998, Haeggman 2010). ESBL production poses a major threat to the use of new cephalosporin generation (Mendes et al. 2004). Long hospitalization, diabetes, age more than 60 and past antibiotic treatment have been accounted for as the hazard elements to gain infections with ESBL strains (Silva1 2006).

In the current study 29 (26.36%) of the *K.pnumoniae* isolates of totally 110 isolated from clinical specimens , showed ESBL production by PCT test were positive for (62.06% , 18/29) , but this current study different from scientific researches related to ESBL production in south Korea (Jeong et al. 2004) showed that (30%) and in Tehran detected (44.5%) (Feizabadi et al. 2006), Positive ESBL concentrations among medically isolated *K.pneumoniae* clinical samples. Another studies about ESBLs in other countries such as Iraq (62.5%) (Al Janaby and Alhasani 2016), Turkey (57%) (Tasli and Bahar 2005) and Lahore Pakistan about (71%) (Ejaz et al. 2013) consistent with our study. The prevalence rate of ESBL production was very diverse in some different countries depending on infection control system and therapeutic methods, but may be due to different antibiotic policies in each countries and differences in the time of isolates collection. In our study, the most common isolates were 16 isolated from urine, the consideration of urinary tract infection is that if not diagnosed early it will lead to renal failure. Previous studies showed that antibiotics were used prior to use (Kim et al. 2002, Lautenbach et al. 2001), the presence of urinary catheters (Schiappa et al. 1996), and Previous hospitalization and use of β-Lactam antibiotics are dangerous agents that cause infection with *K.pneumoniae* or *E.coli* isolates caused by ESBL (Kim et al. 2002).

In this study, the results were showed that the resistance rate is high for piperacilin (100%), ticarcillin (82.72%) but *K.penumoniae* isolates showed less

resistance to augmentin (41.37%) . In the study carried out by Aljanaby and Alhasani in Najaf (Al Janaby and Alhasani 2016), the rate of resistance to ticarcillin (100%, 32/32) was on approach to the rate of resistance in our current study, But the resistance rate. to augmentin in his study was (93.75%, 30/32), also the rate of resistance in Saudi Arabia to augmentin was (86.4%, 190/220) (Al-Agamy et al. 2009), while in the study conducted by Amiri *et al*. (Amiri et al. 2016) in Iran for *klebsiella* isolates *was* (49%) for augmentin and is consistent with our current study, the other study by Nasehi *et al*. (Nasehi et al. 2010) in Iran also showed that the resistance rate for piperacillin was (56%), which contradicts the present study.

The rate of monobactam (aztreonam) resistance in our study was (79.31%), studies conducted in different countries indicate that the rate of resistance to aztreonam is between the minimum and the highest were (36%, 36/100) (Amiri et al. 2016), (97.7%, 215/220) (Al-Agamy et al. 2009), and (100%, 41/41) (Zhang et al. 2018). The difference in resistance may be due to the numeral of isolates, geographical location and the type of antimicrobial *K.pneumoniae* resistance to meropenem and imipenem is very few were (37.93%, 11/29) and (34.48%, 10/29), respectively in our study, this result is in agreement with results Aljanaby and Alhasani which were MEM (25% , 8/32) and IMP (18.75%, 6/32) (Al Janaby and Alhasani 2016), this was confirmed by Dehshiri et al. (Dehshiri et al. 2018) in Iran that resistance to the isolates *K.pneumonia*e isolated from the UTI were MEM (0%, 0/198) and IMP (1%, 2/198), also the study in Saudi Arabia by Al-Agamy el al. (2009), and Amiri *et al*. (2016), were rate resistance for IMP(0% ,0/220) and (7%, 7/100) respectively. The other investigation show all *K.pneumoniae* isolates were sensitive to imipenem (Seyedpour and Eftekhar 2014), likewise in Russian medical clinics (100%) sensitivity to IMP (Edelstein et al. 2003). In contract with other article, the resistance percentage for IMP (8.3%), this was definite by Ishii *et al*. (Ishii et al. 2005) in Japan that the impenem as an active antibiotic for *K.pneumonia*e treatment .

Imipenem is the best action affording to prior studies for complex infections affected by ESBL production by *K.pneumoniae.* But, there are outcomes of other researches that reverse the results of the recent study to resist the *K.pneumoniae* for impenem , where Zhang et al. *(*2018) showed that the resistance rate was (100%, 41/41), Molana et al. (2011) It was also shown that *K.pneumoniae* strains were stayed resistant to IMP in medical specimens (60%).

The present research showed that the two resistant ratio, three and four group cephalosporins including ceftazidime (58.62%), ceftriaxone (48.27%), cefotaxime (37.93%) and cefepime (34.48%). Further studies show that the resistance rate for the two and three group cephalosporins was great where (Zhang et al. 2018), explained that CAZ CRO were (100%) (Al Janaby and Alhasani 2016), also showed that the *K.pneumonia*e isolates to CAZ (71.87%, 23/32) CRO (87.5%, 28/32) and CTX (97.75%, 30/32). Al-Agamy et al. (2009) also proved that the resistance rate for ceftazidime (95.5%, 210/220) and Cefotaxime (97.7%, 215/220) were also high, which contradicted the findings of this study, but the resistance rate for cefepime was (47.8%, 105/220), which is an approach to our study. However, the other studies proved the opposite, where the rate of resistance to ceftriaxone (35%,12.5%), cefotaxime (41%,15.5%) and ceftzidime (33%,12.5%) respectively (Amiri et al., 2016, Dehshiri et al. 2018).

Likewise, the ceftazidime and cefotaxime resistant rate were (34.7%) and (33.5%) separately (Nasehi et al. 2010), these examinations have nearly indistinguishable outcomes from the discoveries of this investigation. Nevertheless, a study (Pai et al. 2004) identifies all patients in its exploration with broad spectrum cephalosporins, either cefotaxime or ceftazidime, and not cefepime, where it was not available at the Seoul General University Hospital's Medical Research Center, then, as it might be, more research is required to determine whether cefepime can be used in the treatment of plasmid AmpC β-lactamase manufacturers. This is showed by the study (Yan et al. 2002), that FEP might be useful for treating infections formed by AmpC β-lactamase-producing organisms.

The discoveries of this examination offered the beta-lactamase genes of the ten isolates for *K.pneumoniae*, which was too the product of β-lactamase and resistance to antibiotics, where the existence of genes were the resulting in this study: βla_{TEM} (80%) $\beta la_{CTX\text{-}M}$ (100%) , βla_{SHV} (80%) and βla_{AMPC} (100%).

A study by Eftekhar *et al*. in 2012 showed that the prevalence rate bla_{TEM} (2.35%) , $\beta la_{CTX\text{-}M}$ (3.31%) and βla_{SHV} (1.43%) in urinary *K.pneumoniae* strains Eftekhar et al. 2012). Leila *et al*. reported the genes isolation rate of βla_{TEM} and βla_{SHV} (18%) and (26%) respectively (Leila et al. 2010). Other studies indicate that the presence of the β-lactamase genes was at a low rate where it was βla_{TEM} (16.1%), $\beta la_{CTM\text{-}1}$ (27.4%) but bla_{SHV} (85.5%) (Dehshiri et al. 2018) , which was higher and this is consistent with our current study , also the other study that prevalence rate of βla_{SHV} , $\beta la_{CTX\text{-}M}$ and βla_{TEM} were (26% , 24.5% , and 18%) respectively (Nasehi et al. 2010, Asif and Mohd 2019).

Most of the studies mentioned above contradict our study. While other reports were indicative of a high prevalence rate of the β-lactamase genes by Al-Agamy et al. (2009) study the SHV, TEM and CTX-M β-lactamase genes (97.3%, 84.1% and 34.1%) respectively, $\beta la_{CTX\text{-}M}$ which are lower compared to the present study. By Faizabadi *et al*. (2010), Aljanaby and Alhasni (2016) the presence of β-lactamase genes was reported about βla_{TEM}, βla_{SHV} (67.4%, 93.75%) and (46.5%, 87.5%) respectively. As well as the study by

Zhang et al. (2018) the most common *β*-lactamase genes were SHV (92.7%, 38/41) followed by TEM (68.3%, 28/41) and CTX-M (43.9%, 18/41) and his study also revealed six isolates carried both ESBL and *AmpC* genes, also study by Amiri *et al*. (2016) was CTX-M (28%) of *klebsiella* isolates carried this gene. Most studies indicate that the presence of CTX-M β-lactamase gene at a small rate, but in the study by kiratisin et al. (1), was CTX-M (99.2%) among ESBL producing *K.penumonia*e isolates, this study is agreement with the current study.

In Pai *et al*. report, the prevalence of AmpC enzyme-producing *K.pneumoniae* it was strong in the National University Hospital in Seoul and the clinical characteristics and results of AmpC-producing species patients were similar to those of TEM or SHV-related producers of ESBL (34).

The resistance to ESBLS could happen by chromosome βla_{SHV} and βla_{TEM} genes are expressed (Mendonca and Ferreira 2009, Abebe et al. 2010). *K.pneumonia*e plasmids contain many bla-genes incoding to AmpC *β*-lactamases, ESBLS, inhibitor resistant, βla_{SHV} and βla_{TEM}, these enzymes make bacteria resistant to many antibiotics including imipenem, meropenem, third generation cepalosporins and others (Essack et al. 2004, Paliy et al. 2019).

In conclusion, the current study result showed that most of *K.pneumoniae* isolates that isolated from different clinical infections were producing beta-lactamases, making them highly resistant to antibiotics especially penicillins, monobactams, second and third generation cephalosporins at the same time. Our study also demonstrated the presence of *β*-lactamase genes encoding TEM, CTX-M, SHV and AmpC enzymes at high rate in the ten isolates that had the least resistance to antibiotics including augmentin, carbapenem, and fourth generation cephalosporins this means that in patients with UTI, burns and respiratory tract infection in Diwaniya hospital, these coded genes pose a serious issue.

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