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Virulence factors and antibiotic susceptibility patterns of multidrug resistance *Klebsiella pneumoniae* isolated from different clinical infections

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Many strains of *Klebsiella pneumoniae* can produce several virulence factors, such as siderophores, capsules, fimbrial adhesins and antibiotic resistance enzymes, which are important in the adherence, colonization, invasion and development of the infection. Therefore the aim of this study was to investigate ten virulence gens in 32 *K. pneumoniae* isolated from different clinical infections: urine (13), burns (12), sputum (4) and blood (3). Phenotypic methods were used to detect capsule, hypermucoviscosity, ability to form biofilm, produce siderophores, primary and confirmatory test of extended spectrum beta lactamase (ESBL). Polymerase chain reaction (PCR) technique was used to detect ten virulence genes encoding attachments (*fimH*, *ycfM*), siderophores (*kfu*: iron uptake system, *entB*: enterobactin, *irp-2*: yersiniabactin), capsule synthesis or invasions (*rmpA*, *uge, wabG*) and beta lactamase enzymes (*SHV*, *TEM*). Antimicrobial susceptibility was tested by disk diffusion method. According to phenotypic methods, the results demonstrated that, the capsule and siderophores production and biofilm formation were observed in 100% of isolates, while hypermucoviscosity phenomenon was observed in 62.5%. The most common virulence genes were *fimH-1*, *ycfM* and *entB* (100%), *uge* and *TEM* (93.75%), *wabG* and *SHV* (87.5%). *Kfu* and *rpmA* genes were found at medium rates of 65.62 and 62.5%, respectively and at lower prevalence was gene *Irp-2* (37.5%). A rate of 84.375% of isolates showed a multidrug resistance (MDR) pattern, 12.5% extensive drug resistance (XDR), 3.12% pandrug resistance (PDR) and the rate of extended-spectrum β- lactamases (ESBL) producing *K. pneumoniae* was 62.5%.The study concluded that *fimH*-1, *ycfM* and *entB* genes were commonly found in all isolates, they seem to be at the basis of classic pathogenicity of *K. pneumoniae*. The most common virulence genes were observed in isolates from burns and blood samples. *K. pneumoniae* became highly resistant to antibiotics especially to 3rd generation cephalosporins and there was positive relationship between presence of virulence genes and ability of the bacterium to antibiotics resistance.

Key words: *Klebsiella pneumoniae*, drug resistance, virulence genes, Iraq.

INTRODUCTION

Klebsiella pneumoniae is one of the most important pathogenic bacteria. It is gram negative, bacilli, non-motile and causative agent of many diseases, such as pneumonia, urinary tract infections, bacteremia, burns

and wounds infections and pyogenic liver abscesses (Rahamathulla et al., 2016). Pathogenicity of *K. pneumoniae* is due to the presence of many virulence genes which encode virulence factors that allow it to attack the immune system of mammalians and cause many kind of diseases. Some of these virulence factors are: biofilm formation, hypermucoviscosity, capsule synthesis, adhesions, iron uptake and lipopolysaccharides formation (Fertas-Aissani et al., 2013; Chung et al., 2015). *K. pneumoniae* has been found capable to resist many antibiotics especially third generation cephalosporins like cefotaxime, ceftriaxone and ceftazidime (Yeh et al., 2007). Many clinical features of *K. pneumoniae* infections are related with virulence gens according to number and mode of action of these genes (Wiskur et al., 2008). Recently *K. pneumoniae* is found causing acute liver abscess as reported in many Asian countries like China, Kuwait and Iraq (Christopher et al., 2014; Chung et al., 2015). *K. pneumoniae* mostly contains extended spectrum beta-lactamase genes (*SHV, TEM* and *C-TXM*) that encoded by plasmid. These genes have shown resistance to many types of antibiotics (more than three classes) which is considered as multidrug resistant bacteria (Ahmed et al., 2014). In spite of *K. pneumoniae* is considered as one of the most important opportunistic bacteria, knowledge of the mechanism by which this bacterium causes many diseases is still not fully understood. The aim of this study was to investigate the presence of virulence genes in 32 *K. pneumoniae* clinical isolates from different clinical sources.

MATERIALS AND METHODS

Bacterial isolates

This study was carried out in Al-Sadder Medical City in AL-Najaf Governorate-Iraq. A total of 464 specimens from different clinical sources were collected from patients with (urinary tract infections, respiratory tract infections, burns infections and blood infections) during the period of January to October 2015. All urine and sputum samples were collected in sterile containers, a loopful of the urine and sputum samples were streaked onto the surface of MacConkey agar and blood agar (Oxoid,UK) and incubated at 37°C for 24 h. Burns samples were collected by sterile swabs and streaked onto the surface of MacConkey agar and blood agar (Oxoid, UK) and incubated at 37°C for 24 h. Five milliliter of blood samples were collected by a sterile syringes and mixed with 45 ml of brain heart infusion broth (Oxoid, UK) and incubated at 37°C for 7 days , and streaked (by sterile loop) onto the surface of blood and MacConkey agar (Oxoid, UK) and incubated at 37°C for 24 h (Collee et al.,1996). All isolates were identified according to morphological and biochemical tests (Gram stain, Growth on MacConkey Agar, Capsule stain, Catalase and oxidase test, Urease production test, Motility test, Indole production test, Methyl Red test, Voges-Proskauer test, Simmons Citrate test and Triple Sugar Iron test) (MacFaddin, 2000) and then by cultivation on Chrome agar medium (Orientation company – France). Finally all suspected isolates of *K. pneumoniae* were identified by using Vitek2® system (BioMerieux® -France).

Phenotypic determination of virulence factors

Capsule detection

The presence of capsule was investigated by staining with nigrosin, a loopful of overnight bacterial colony was transferred on a dry and clean slide, then gently mixed with nigrosin and allowed to dry in air, then rinsed with water , the slide was stained with methylen blue for 2 min and allowed to air dry, then the slide was gently washed with water, under light microscope the nigrosin stain provides a dark background to unstained capsule and metylene blue stain provides blue color to the cells (Soensen, 1995).

Hypermucoviscosity testing

Single colonies after culturing on Brain Heart Infusion agar plates (Oxoid, UK) were obtained and tested for their ability to form viscous strings. When a standard inoculation loop was touched onto their surface and slowly raised. The formation of string greater than 5 mm in length is indicative of hypermucoviscosity positive phenotype (Grange, 1988).

Siderophores production assay

Nutrient agar supplemented with 200 mM of 2.2'-dipyridyl was used as iron-restricted agar medium. All bacterial isolates were streaked on agar plates, and then incubated at 37°C for 24 h. Any bacterial growth was considered as positive results for ability of bacteria to siderophores production (Schwyn and Neilands, 1987).

Biofilm formation testing

Briefly, *K. pneumoniae* isolates were sub cultured three times in LB broth for 18 h at 37°C. The optical density was adjusted to 0.55 to 0.65 (2.106 to 8.109 CFU/mL) at 540 nm. Two hundred milliliter of the adjusted bacterial cultures were transferred to 96-well polystyrene microtiter plates and incubated for 24 h at 25°C. After addition of 25 mL of 1% crystal violet to each well, the plate was shaken and incubated for 15 min at room temperature. After removal of medium and three washes with phosphate-buffered saline, crystal violet was dissolved by the addition of ethanol, and the absorbance (Izquierdo et al., 2003).

DNA extraction

DNA was extracted by a boiling method as follows: Three to five pure and fresh colonies were suspended in 300 µl of distilled water, then cells were lysed by heating at 100°C for 20 min (in water bath), immediately the cells were placed in ice for 30 mi and the other cellular components was removed by centrifugation at 8500

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Table 1. Primers used in the current study.

Target genes	Primers'sequences (5′ to 3′)	Product size (bp)	References
entB	F-ATTTCCTCAACTTCTGGGGC R-AGCATCGGTGGCGGTGGTCA	371	(Fertas et al*.,* 2013)
irp-2	F-TCCCTCAATAAAGCCCACGCT R- TCGTCGGGCAGCGTTTCTTCT	287	(Schubert et al.,2000)
kfu	F-GAA GTG ACG CTG TTT CTG GC R-TTT CGT GTG GCC AGT GAC TC	797	(Ma et al., 2005)
rpmA	F-ACTGGGCTACCTCTGCTTCA R-CTTGCATGAGCCATCTTTCA	535	(Yeh et al.,2007)
wabG	F-CGGACTGGCAGATCCATATC R-ACCATCGGCCATTTGATAGA	683	(Brisse et al., 2009)
uge	F-TCT TCA CGC CTT CCT TCA CT R-GAT CAT CCG GTC TCC CTG TA	534	(Regue et al., 2004)
Fimh1	F-ATGAACGCCTGGTCCTTTGC R-GCTGAACGCCTATCCCCTGC	688	(Fertas et al*.,* 2013)
ycfm	F-ATCAGCAGTCGGGTCAGC R-CTTCTCCAGCATTCAGCG	160	
SHV	F- GGCCGCGTAGGCATGATAGA R- CCCGGCGATTTGCTGATTTC	714	(Ensor et al., 2009)
TEM	F- CAGCGGTAAGATCCTTGAGA R- ACTCCCCGTCGTGTAGATAA	643	

rpm for 10 min. Finally the supernatant was used as the DNA template (Yang et al., 2008).

PCR detection of virulence-associated genes

PCR was used to detect genes encoding type 1 adhesins (*fimH*-1), outer membrane lipoprotein (*ycfM*), enterobactin biosynthesis (*entB*), yersiniabactin biosynthesis (*irp-2*), iron uptake system (*kfu*), regulator of mucoid phenotype A (*rmpA*), invasions (*uge, wabG*) and beta lactamase enzymes (*SHV* , *TEM*). All primers used are listed in Table 1. And polymerase chain reaction (PCR) thermo cycling conditions are listed in Table 2.

Antimicrobial susceptibility testing

Antibiotic susceptibility testing was performed by the Kirby-Bauer method according to the Clinical Laboratory Standards Institute (CLSI, 2014). A colony from each *K. pneumoniae* isolate was grown overnight in Mueller Hinton broth (Oxoid,UK) at 37°C. Bacterial cultures were adjusted to 0.5 on the MacFarland nephelometer scale (1.5×10^8 CFU/ml) and plated on Mueller Hinton agar (Oxoid, UK) by the streaking method using a sterile swab. Antimicrobial susceptibility and resistance was determined by isolate growth zone diameter according to CLSI guidelines (2014). E. *coli* ATCC 25922 strain was used as controls. Antibiotics disks were purchased from Bioanalyse, Turkey. All types of antibiotics are listed in Table 3.

Primary test for production of ESBL

Antibiotic susceptibility testing was done to three types of 3rd generation cephalosporins antibiotics: ceftazidime, cefotaxime, and ceftriaxone. If inhibition zone for bacterial isolates were: ≤27 mm for cefotaxime, ≤22 mm for ceftazidime and ≤25 mm for ceftriaxone, this result considered as positive result for production of extended spectrum beta lactamase (CLSI, 2006).

Confirmatory test for ESBL

Augmentin disc 30 µg was placed in the center of Mueller Hinton agar plate (Oxoid, UK). Around of three sides of augmentin disc (30 µg), a disc of ceftazidime (30 µg), ceftriaxone (30 µg) and cefotaxime (30 µg) were placed with distance of 15mm from center to center of augmentin disc. Then the plate was incubated at 37°C for 24 h.

If inhibition zone was increased towards the augmentin disc that considered as positive results for production of ESBL (Sarojamma and Ramakrishna, 2011).

Table 2. PCR thermo cycling conditions.

Gene	Initial Denaturation °C / Time	Denaturation °C / Time	Annealing °C / Time	Extension °C / Time	Number of Cycles	Final extension °C / Time	References
entB	94°C /4 min	94°C /30 s	57°C /40 s	72°C /60 s	30	72°C /10 min	(Fertas et al., 2013)
irp-2	94°C /4 min	94°C /30 s	57°C /40 s	72°C /60 s	30	72 °C /10 min	(Schubert et al.,2000)
kfu	94°C /5 min	94°C /60 s	54°C /45 s	72°C /60 s	35	72 °C /7 min	(Ma et al., 2005)
rpmA	94°C /4 min	94°C /30 s	50 °C /40 s	72°C /60 s	30	72 °C /10 min	(Yeh et al.,2007)
uge	94°C /5 min	94°C /60 s	54°C /45 s	72°C /60 s	35	72 °C /7 min	(Brisse et al., 2009)
wabG	95°C /3 min	94°C /30 s	53°C /30 s	72°C /60 s	30	72 °C /5 min	(Regue et al., 2004)
fimH-1	94°C /4 min	94°C /30 s	55 °C /40 s	72°C /60 s	30	72 °C /10 min	(Fertas et al., 2013)
ycfM	94°C /4 min	94°C /30 s	55 °C /40 s	72°C /60 s	30	72°C /10 min	
TEM	95°C /5 min	94°C /30 s	52°C /45 s	72°C /45 s	30	72 °C /7 min	(Ensor et al., 2009)
SHV	95°C /5 min	94°C /30 s	55°C /60 s	72°C /45 s	30	72 °C /7 min	

Table 3. Antibiotic discs used in the current study.

Antibiotic	Symbol	Concentration (µg)
Amoxicillin	AX	25
Ticarcillin	TIC	75
Amoxicillin + Clavulanic acid	AMC	30
Cefotaxime	CTX	30
Ceftriaxone	CRO	30
Ceftazidime	CAZ	30
Imipenem	IMP	10
Meropenem	MEM	10
Gentamicin	CN	10
Amikacin	AK	30
Tobramycin	TM	10
Tetracycline	TE	30
Doxycycline	DO	30
Ciprofloxacin	CIP	5
Chloramphenicol	C	30
Nitrofurantoin	F	300

Statistical analysis

Statistical analysis was performed with GraphPad Prism version 5 software, percentages was used for the comparison between samples of the study.

RESULTS

K.pneumoniae isolates

Out of the 464 total specimens there were 243 specimens (52.370%) it has been diagnosed as gram negative bacteria, while there were 196 specimens (42.241%) were gram positive bacteria and 25 specimens (5.389%) with no any growth (Table 4). A total of 243 g negative bacterial isolates were collected from different clinical sources (106 from urine, 90 from burns, 25 from sputum and 22 from blood) there were 13 isolate of *K. pneumoniae* (12.264%) from urine, 12 isolates (13.333%) from burns, 4 isolates (16%) from sputum and only 3 isolates (13.636%) from blood (Table 5).

Virulence factors

In the present study, phenotypic methods were used to detect virulence factors, the capsule, biofilm formation and siderophores productions were observed in 32 isolates (100%), hypermucoviscosity phenomenon was observed in 20 isolates (62.5%) (Figure 1). Out of total 32

Table 4. Numbers and percentage of total samples (N=464).

Bacterial growth	No.	Percentage
Gram negative	243	52.370
Gram positive	196	42.241
No growth	25	5.389
Total	464	100

Table 5. Numbers and percentage of *K. pneumoniae* according to total gram negative bacterial isolates and sites of infection (N= 243).

Site of infection	Numbers of Gram negative bacteria	*K.pneumoniae*	Percentage (100%)
Urine	106	13	12.264
Burns	90	12	13.333
Sputum	25	4	16
Blood	22	3	13.636
Total	243	32	13.168

Figure 1. Stretching of *K. pneumoniae* colonies isolated from patient with blood infection resulted in the formation of a string >5 mm in length, demonstrating the hypermucoviscosity phenotype.

isolates, 32 isolates (100%) were positive for *fimh1*, *ycfm* and *entB* genes (Figures 2, 3 and 4, respectively), 30 isolates (93.75%) were positive for *uge* and *TEM* genes (Figures 5 and 6 , respectively), 28 isolates (87.5%) were positive for *wabG* and *SHV* genes (Figures 7 and 8, respectively), 21 isolates (65.62%) were positive for *kfu* gene (Figure 9), 20 isolates (62.5%) were positive for *rpmA* gene (Figure 10) and 12 isolates (37.5%) were positive for *Irp-2* gene (Figure 11). The prevalence and distribution of virulence factors were given in Tables 6 and 7.

Antimicrobial susceptibility

All isolates of *K. pneumoniae* showed resistance to AX, TIC and F (100%, 32/32), AMC and CTX (97.75%, 30/32), CRO (87.5%, 28/32), CAZ (71.875% 23/32), CIP

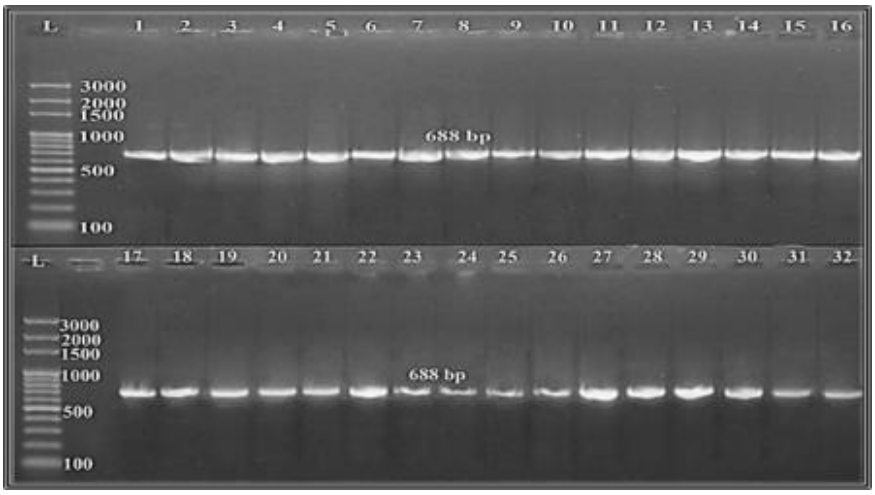

Figure 2. Ethidium bromide-stained agarose gel electrophoresis of monoplex PCR amplified products from extracted total DNA of *klebsiella pneumoniae* . lane: (1 to 32 isolates) amplified with diagnostic *fimH-1* gene, show positive results at 688 bp. The electrophoresis was performed at 80 volt for 95 min.

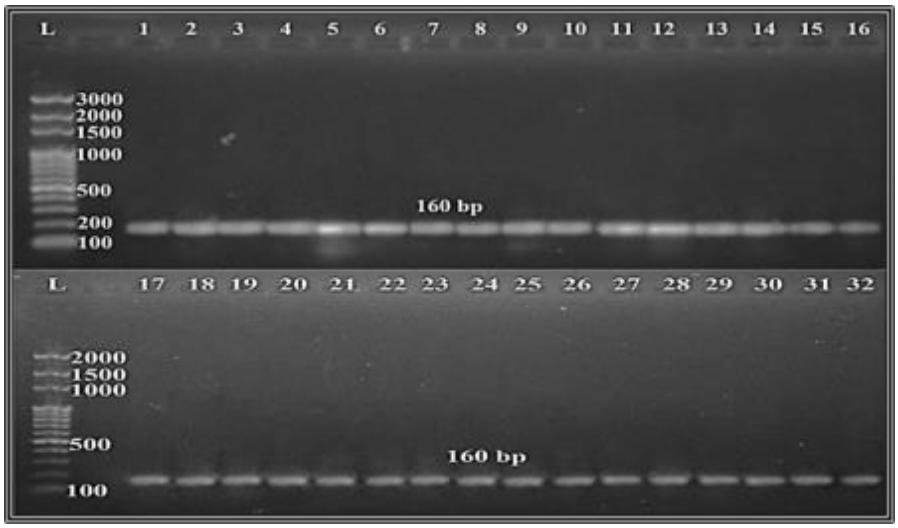

Figure 3. Ethidium bromide-stained agarose gel electrophoresis of monoplex PCR amplified products from extracted total DNA of *klebsiella pneumoniae*. Lane: (1 to 32 isolates) amplified with diagnostic *ycfM* gene, show positive results at 160 bp . The electrophoresis was performed at 80 volt for 95 min.

(43.75%, 14/32), DO (40.62%, 13/32), TM and TE (34.37, 11), CN and C (31.25%, 10/32), AK (28.12% , 9/32), MEM(25% , 8/32) and IMP (18.75% 6/32). Also in this study, 84.37% (27/32) of *K. pneumoniae* isolates were MDR, 12.5% (4/32) were XDR and 12% (1/32) were PDR. Both primary and confirmatory test for ESBL production were positive for 62.5% (20/32) of the isolates resistant to third-generation cephalosporins (Figures 12 and 13). Resistance test to 16 antibiotic of *K. pneumoniae* isolates were given in Tables 8, 9 and 10.

DISCUSSION

The genes *fimH*-1 and *ycfm* were found in all of our isolates. This result is in agreement with the ubiquitous nature of this structure in *K. pneumoniae* as reported in Fertas-Aissani et al. (2013). Microorganisms with biofilm formation are associated with many human infections. The biofilm formation role and development by bacteria has been documented to be a crucial step in the pathogenesis of *Klebsiella*. The flushing action of sterile

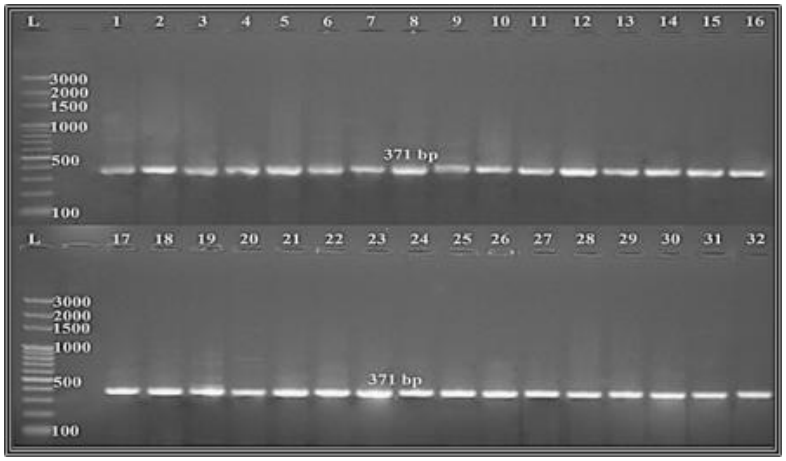

Figure 4. Ethidium bromide-stained agarose gel electrophoresis of monoplex PCR amplified products from extracted total DNA of *klebsiella pneumoniae*. Lane: (1 to 32 isolates) amplified with diagnostic *entB* gene, show positive results at 371 bp . The electrophoresis was performed at 80 volt for 95 min.

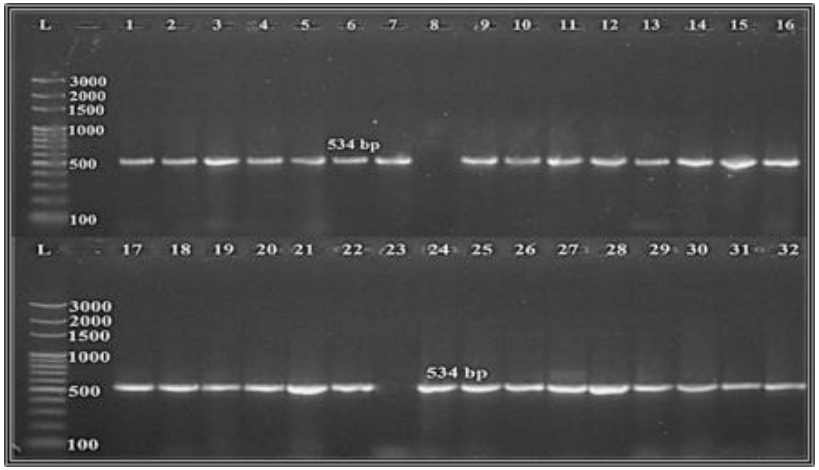

Figure 5. Ethidium bromide-stained agarose gel electrophoresis of monoplex PCR amplified products from extracted total DNA of *klebsiella pneumoniae*.Lane: (1 to 32 isolates) amplified with diagnostic *uge* gene, show positive results at 534 bp .The electrophoresis was performed at 80 volt for 95 min.

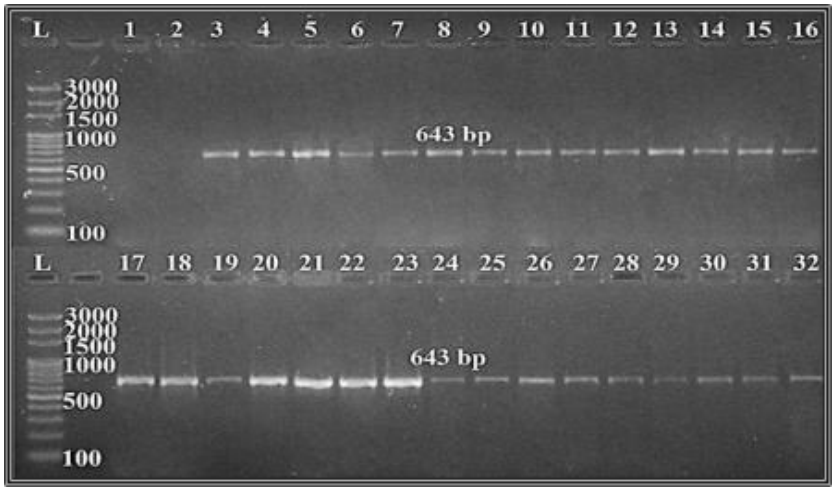

Figure 6. Ethidium bromide-stained agarose gel electrophoresis of monoplex PCR amplified products from extracted total DNA of *klebsiella pneumoniae* . Lane: (1 to 32 isolates) amplified with diagnostic *TEM* gene, show positive results at 643 bp . The electrophoresiswas performed at 80 volt for 95 min.

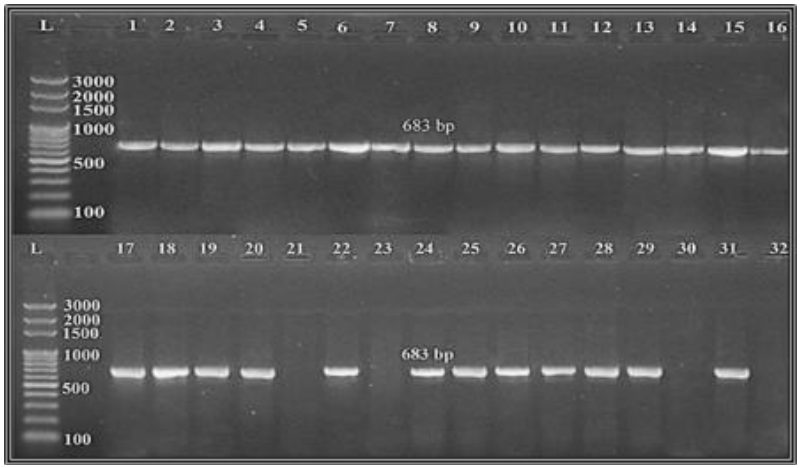

Figure 7. Ethidium bromide-stained agarose gel electrophoresis of monoplex PCR amplified products from extracted total DNA of *klebsiella pneumoniae*. lane: (1 to 32 isolates) amplified with diagnostic *wabG* gene, show positive results at 683 bp . The electrophoresis was performed at 80 volt for 95 min.

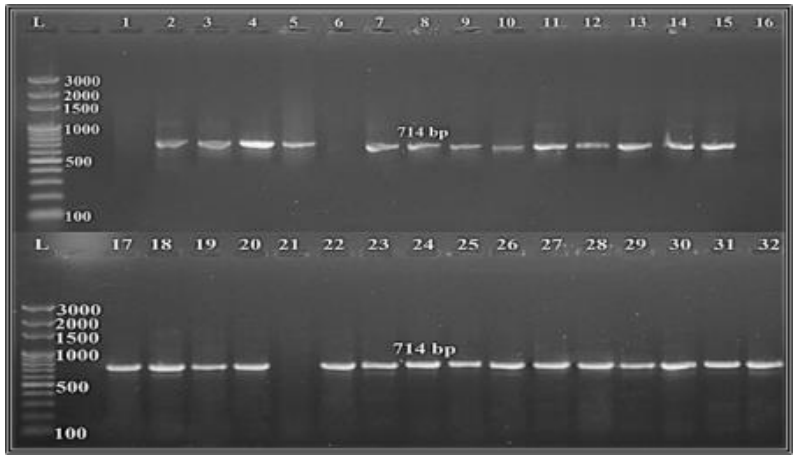

Figure 8. Ethidium bromide-stained agarose gel electrophoresis of monoplex PCR amplified products from extracted total DNA of *klebsiella pneumoniae*. Lane: (1 to 32 isolates) amplified with diagnostic *SHV* gene, show positive results at 714 bp. The electrophoresis was performed at 80 volt for 95 min.

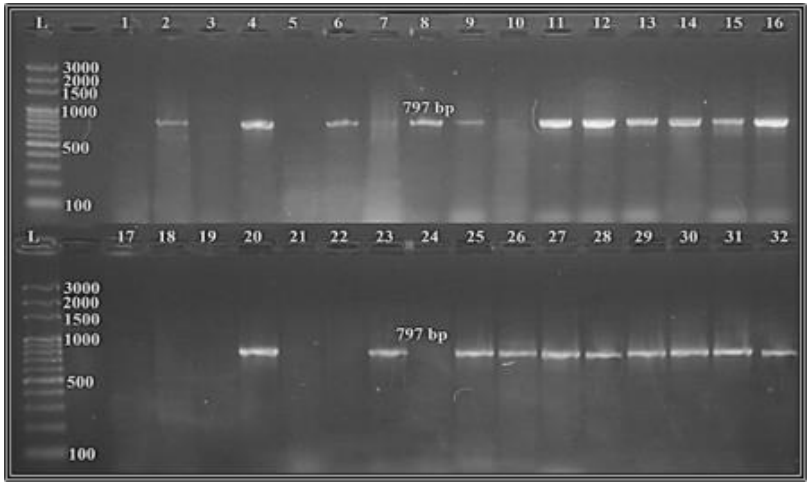

Figure 9. Ethidium bromide-stained agarose gel electrophoresis of monoplex PCR amplified products from extracted total DNA of *klebsiella pneumoniae*. Lane: (1 to 32 isolates) amplified with diagnostic *kfu* gene, show positive results at 797 bp. The electrophoresis was performed at 80 volt for 95 min.

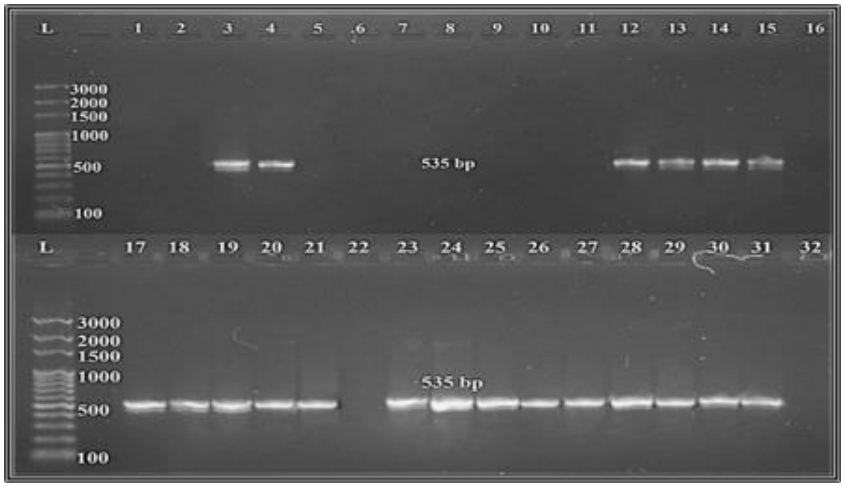

Figure 10. Ethidium bromide-stained agarose gel electrophoresis of monoplex PCR amplified products from extracted total DNA of *klebsiella pneumoniae*. Lane: (1 to 32 isolates) amplified with diagnostic *rpmA* gene, show positive results at 535 bp. The electrophoresis was performed at 80 volt for 95 min.

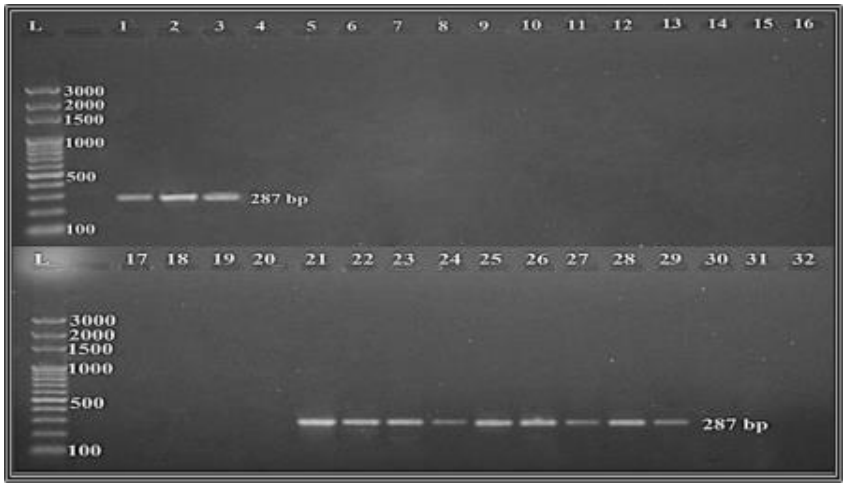

Figure 11. Ethidium bromide-stained agarose gel electrophoresis of monoplex PCR amplified products from extracted total DNA of *klebsiella pneumoniae*. Lane: (1 to 32 isolates) amplified with diagnostic *irp-2* gene, show positive results at 535 bp. The electrophoresis was performed at 80 volt for 95 min.

Figure 12. Confirmatory test for ESBL of *Klebsiella pneumoniae* by Double Disc Synergy Test. Ceftazidime (CAZ 30 µg) ; Ceftriaxone (CRO 30 µg); Amoxi/Clavulanic acid (AMC 30 µg); Cefotaxime (CX 30 µg)

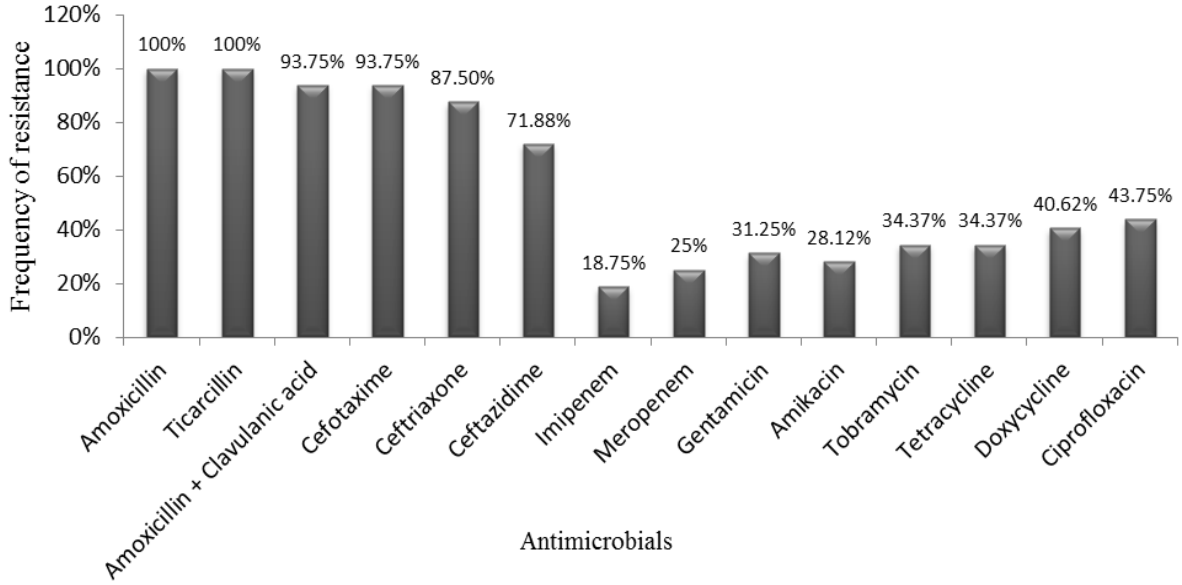

Figure 13. Frequency of antimicrobial resistance to *Klebsiella pneumonia.*

urine in urinary tract is protected from colonization by pathogen, but formation of biofilms by bacteria in urinary tract was lead to infect by chronic urinary tract infections. Biofilms and outer membrane lipoproteins can play an important role in protecting bacteria from drugs exposure when compared with other bacteria do not have these virulence factors (Bellifa et al.,2013). Therefore, there was strong relationship between resistance of antibiotics and prevalence of these virulence factors in bacteria. Bacteria with biofilm forming are generally more resistant

Table 6. Prevalence and distribution of virulence factors according to clinical samples (n = 32).

Virulence factors n (%)										
PCR amplification										
	Adhesins		Siderophores			Biosynthesis of capsule / invasins			ESLB	
Samples	***Fimh-1***	***ycfm***	***Entb***	***irp-2***	***kfu***	***RmpA***	***wabG***	***uge***	***TEM***	***SHV***
Urine (n=13)	13	13	13	3	7	5	9	11	12	11
Burns (n=12)	12	12	12	4	8	11	12	12	12	11
Sputum (n=4)	4	4	4	2	3	1	4	4	3	3
Blood (n=3)	3	3	3	3	3	3	3	3	3	3
Total / 32	32	32	32	12	21	20	28	30	30	28
	(100)	(100)	(100)	(37.5)	(65.6)	(62.5)	(87.5)	(93.7)	(93.7)	(87.5)

Virulence factors n (%)									
Phenotypic determination									
	BF	CPS	HMV	Siderophores	Antibiotics resistance pattern			ESBL	
Samples					**MDR**	**XDR**	**PDR**	**Pri.t**	**Conf.t**
Urine (n=13)	13	13	5	13	10	0	0	8	8
Burns (n=12)	12	12	8	12	10	1	0	8	8
Sputum (n=4)	4	4	4	4	4	0	0	1	1
Blood (n=3)	3	3	3	3	3	3	1	3	3
Total / 32	32	32	20	32	27	4	1	20	20
	(100)	(100)	(62.5)	(100)	(84.3)	(12.5)	(12)	(62.5)	(62.5)

ESLB: extended spectrum beta lactamase; *fimh-1*: FimH-like adhesin ; *ycfM*: outer membrane lipoprotein; *Entb*: enterobactin; *irp-2*: yersiniabactin; *kfu*: iron uptake system; *rmpA*: regulator of mucoid phenotype; *wabG*: lipopolysaccharides- core gene; *uge*: uridine diphosphate galacturonate 4-epimerase; *TEM*: β-lactamase named after first patient isolated from Temarian; *SHV*: Sulfhydryl variable β-lactamas; BF: biofilm formation; CPS: capsule; MHV: hypermucoviscosity ; MDR: Multidrug resistance; XDR:Extensive drug resistance; PDR: Pandrug resistance; Pri.t: primary test; conf.t: onfirmatory test.

Table 7. Numbers and percentage of virulence genes among 32 *K. pneumoniae* isolates according to site of infection.

Gene	Urine Total isolates: 13	Burns Total isolates: 12	Sputum Total isolates: 4	Blood Total isolates: 3	Total (32)
Entb	13(100%)	12(100%)	4(100%)	3(100%)	32(100%)
Irp-2	3(23.07%)	4(33.33%)	2(50%)	3(100%)	12(37.5%)
Kfu	7(53.84%)	8(66.66%)	3(75%)	3(100%)	21(65.62%)
Fimh1	13(100%)	12(100%)	4(100%)	3(100%)	32(100%)
Ycfm	13(100%)	12(100%)	4(100%)	3(100%)	32(100%)
wabG	9(76.92%)	12(100%)	4(100%)	3(100%)	28(87.5%)
rpmA	5(38.46%)	11(91.66%)	1(25%)	3(100%)	20(62.5%)
Uge	11(84.61%)	12(100%)	4(100%)	3(100%)	30(93.75%)
TEM	12(92.30%)	12(100%)	3(75%)	3(100%)	30(93.75%)
SHV	11(84.61%)	11(91.66%)	3(75%)	3(100%)	28(87.5%)

to many antibiotics. Biofilms and outer membrane lipoproteins act as biodegradable effect on beta-lactamases antibiotics. Beta-lactamase enzymes are secreted and maintain their activity inside of biofilm matrix, and decompose beta-lactam antibiotics before these antibiotics reach the bacterial cells (Høiby et al., 2010). Antibiotics penetration inside of biofilms could be blocked by other factors, such as the presence of surfaces with negatively-charged, particularly for large polar molecules such as aminoglycosides antibiotics with positively charged, Also, trace amount of metabolic rates and limited oxygen are maybe important factors contributing to increasing resistance to fluoroquinolones, cephalosporins, aminoglycosides and beta-lactamase

Table 8. Antibiotics sensitivity test for 32 clinical isolate of *K. pneumonia.*

Antibiotics	Concentration	Sensitive	Intermediate	Resistant	Percentage of resistance (%)
Amoxicillin	25 µg	0	0	32	100
Ticarcillin	75 µg	0	0	32	100
Amoxicillin + Clavulanic acid	20+10 µg	2	0	30	93.75
Cefotaxime	30 µg	2	0	30	93.75
Ceftriaxone	30 µg	4	0	28	87.5
Ceftazidime	30 µg	9	0	23	71.875
Imipenem	10 µg	23	3	6	18.75
Meropenem	10 µg	23	1	8	25
Gentamicin	15 µg	22	0	10	31.25
Amikacin	30 µg	20	3	9	28.12
Tobramycin	10 µg	19	2	11	34.37
Tetracycline	30 UI	20	1	11	34.37
Doxycycline	30 µg	19	0	13	40.62
Ciprofloxacin	5 µg	16	2	14	43.75
Chloramphenicol	30 µg	22	0	10	31.25
Nitrofurantoin	30 µg	0	0	32	100

antibiotics (Kwon et al., 2008; Lewis, 2001).

Siderophores are bio-synthetically compounds produced and secreted by many microorganisms such as, fungi and bacteria for iron (Fe^{+3}) uptake, and they are selective chelators. These ions are weakly soluble and almost found in all oxygenated environments. In this study, *Entb* gene was found in all our isolates (100%, 32/32), while *irp-2* gene was found in some of our isolates (37.5%, 12/32). These results were in agreement with some previous studies showed that almost all *K. pneumoniae* clinical isolates were having these chelators (Podschun et al., 1993; Koczura and Kaznowski, 2003; Fertas-Aissani et al., 2013) also in this study, *kfu* gene was found in 21 isolates (65.62%). Enterobactin and yersiniabactin they are siderophore compounds produced by bacterial cells to uptake iron (Fe^{+3}) from iron-binding proteins of the host, and they are have strong-affinity extracellular ferric chelators, which; they have an important role in pathogenesis and virulence of bacteria, virulence of these compounds are remain unclear; however, the expression of enterobactin induced biofilm formation (May and Okabe, 2011). But Lai et al. (2001) suggested that the expression of gene for iron-enterobactin that responsible for outer membrane receptor appears to be activated during bacterial infection.

The *kfu* gene is a putative pathogenic gene which codes for an iron uptake system. *kfu* gene associated with the purulent tissue infections ,capsule formation and virulent hypermucoviscosity phenotype. Therefore, this gene is considered as a very important gene in iron up take from host cell (Aher et al., 2012).

In this study, all our isolates had a capsule, while only 62.5% (20/32) had a hypermucoviscosity phenotype and *rpmA* gene. Almost *K. pneumoniae* produce large amounts of muco-polysaccharide mass and extra-capsular polysaccharides to produce strain with more virulent (Wiskur et al., 2008). In line with this Victor et al. (2007) reported that mucoid phenotype was seen in all isolates of *K. pneumoniae* that caused the invasive syndrome and in more than 90% of isolates in human with community- acquired pneumonia and in South Africa and Taiwan. Also he found that mortality of laboratory animals injected with mucoid strains was higher than that occurring in the same laboratory animals injected with non-mucoid strains.

The *rmpA* gene is a main factor that has an important role in virulence of *K. pneumoniae* strains, is controlled by plasmid with strongly mucoviscous phenotype promoted and synthesis regulator of the capsular polysaccharide (Rivero et al., 2010). The results demonstrated that out of total 32 isolates, 30 isolates (93.75%) were positive for *uge* gene and 28 isolates (87.5%) were positive for *wabG* gene.

wabG gene is less virulence factor studied, although it has been observed that many of *wabG* gene positive strains are from the patients with invasive and serious infections. But the mechanism of mode of action is still unclear (Turton et al., 2010). Izquierdo et al. (2003) proved that strains of *K. pneumoniae* with mutant *wab G* gene were non capsulated and less virulent in murine pneumonia model (rats). This fact proves the important role of *wab G* gene in pathogenicity of *K. pneumoniae*. Because the defect in synthesis of core lipopoly-saccharides in strains of *K. pneumoniae* with mutant *wab-G* gene were unable to induce urinary tract infection in experimental rats. Since they were unable to colonize in urinary tract in comparison with that of the normal

Table 9. Profile of virulence genes and resistance pattern among 32 clinical isolates of *K. pneumoniae* according to site of infection

No. of isolate	samples	*Entb*	*irp2*	*Kfu*	*FimH1*	*Ycfm*	*wabG*	*rpmA*	*Uge*	*TEM*	*SHV*	Phenotypic detection
1	Urine	+	+	-	+	+	+	-	+	-	-	
2	Sputum	+	+	+	+	+	+	-	+	-	+	
3	Burns	+	+	-	+	+	+	+	+	+	+	
4	*Burns	+	-	+	+	+	+	+	+	+	+	
5	*Urine	+	-	-	+	+	+	-	+	+	+	
6	Sputum	+	-	+	+	+	+	-	+	+	-	
7	*Urine	+	-	-	+	+	+	-	+	+	+	
8	*Urine	+	-	+	+	+	-	-	-	+	+	
9	*Urine	+	-	+	+	+	+	-	+	+	+	
10	*Urine	+	-	-	+	+	+	-	+	+	+	
11	*Burns	+	-	+	+	+	+	-	+	+	+	
12	*Burns	+	-	+	+	+	+	+	+	+	+	
13	*Burns	+	-	+	+	+	+	+	+	+	+	
14	*Sputum	+	-	+	+	+	+	+	+	+	+	MDR
15	*Burns	+	-	+	+	+	+	+	+	+	+	
16	Urine	+	-	+	+	+	+	-	+	+	-	
17	Burns	+	-	-	+	+	+	+	+	+	+	
18	Burns	+	-	-	+	+	+	+	+	+	+	
19	Urine	+	-	-	+	+	+	+	+	+	+	
20	Burns	+	-	+	+	+	+	+	+	+	+	
21	Urine	+	+	-	+	+	-	+	+	+	-	
22	*Sputum	+	+	-	+	+	+	-	+	+	+	
23	*Urine	+	+	+	+	+	+	+	-	+	+	
24	*Burns	+	+	-	+	+	+	+	+	+	+	
25	*Blood	+	+	+	+	+	+	+	+	+	+	PDR
26	*Burns	+	+	+	+	+	+	+	+	+	+	
27	*Blood	+	+	+	+	+	+	+	+	+	+	
28	*Blood	+	+	+	+	+	+	+	+	+	+	XDR
29	*Burns	+	+	+	+	+	+	+	+	+	+	
30	*Urine	+	-	+	+	+	-	+	+	+	+	
31	Urine	+	-	+	+	+	+	+	+	+	+	MDR
32	Urine	+	-	+	+	+	-	-	+	+	+	

MDR: Multidrug resistance, XDR: Extensive drug resistance, PDR: Pandrug resistance, *: Extended spectrum β-lactamase. +: present, -: absent.

strains. But the mutant bacterial strains were still able to perform some colonization. Regué et al. (2004) proved that the mutant strains of *K. pneumoniae* (without *uge* gene) were non-virulent in laboratory animals; this fact proves the important role of *uge* gene in pathogenicity of *K. pneumoniae*.

The antibiotic susceptibility analysis of the isolates showed that 100% (32/32) were resistance to amoxicillin, ticarcillin and nitrofurantoin. On the other hand, 84.37% (27/32) of isolates were MDR, 12.5% (4/32) were XDR and 12% (1/32) were PDR. The presumptive test for ESBL production was positive for 65.5% (20/32). The genes *TEM* and *SHV* were 93.75% (30/32) and 87.5% (28/32). These resistance patterns can be due to the expression of different enzymes such as extended-spectrum beta lactamases. Multi-drug resistances are often associated with extended spectrum beta-lactamase producing bacteria, that is, resistance to other classes of drugs like aminoglycosides and quinolones (Roshan et al., 2011). Isolates of *K. pneumoniae* may be have natural resistance to amoxicillin, ampicillin and ticarcillin but not to ESBL antibiotics. The resistance to Extended Spectrum Beta-Lactamase could happen class A chromosome beta-lactamase *SHV* and *TEM* genes are expressed (Mendonca and Ferreira, 2009). *K. pneumoniae* plasmids contain many B-lactamase genes including those encoding AmpC beta-lactamases, extended-spectrum B-lactamases, inhibitor resistant, metallo enzymes, *SHV* and *TEM* beta-lactamases. These enzymes make bacteria enable to resistant to different

Table 10. Sensitivity test and type of resistant among 32 clinical isolates of *K. pneumoniae* to 16 types of antibiotics according to site of infection.

No. of isolate	Sours of samples	Antibiotics																Phenotypic detection
		AX	TIC	AMC	CTX	CRO	CAZ	IMP	MEM	CN	AK	TM	TE	DO	CIP	C	F	
1	Urine	R	R	S	S	S	S	S	S	S	S	S	R	S	S	R	R	
2	Sputum	R	R	R	R	S	R	I	R	R	R	R	S	R	R	R	R	
3	Burns	R	R	R	R	R	R	R	R	R	R	R	S	R	R	S	R	
4	*Burns	R	R	R	R	R	R	S	S	S	S	S	S	R	S	S	R	
5	*Urine	R	R	R	R	R	S	S	S	S	S	R	R	S	S	S	R	
6	Sputum	R	R	R	R	R	R	S	S	S	S	S	S	R	R	S	R	
7	*Urine	R	R	R	R	R	S	S	S	S	S	S	R	S	S	R	R	
8	*Urine	R	R	R	R	R	S	S	S	S	S	S	R	R	S	R	R	
9	*Urine	R	R	R	R	R	S	S	S	S	S	S	S	R	S	S	R	
10	*Urine	R	R	R	R	R	S	S	S	S	S	S	S	S	S	S	R	
11	*Burns	R	R	R	R	R	R	S	S	S	S	S	R	S	S	S	R	
12	*Burns	R	R	R	R	R	R	I	R	R	R	R	S	R	R	S	R	
13	*Burns	R	R	R	R	R	R	S	S	S	S	S	S	R	S	S	R	MDR
14	*Sputum	R	R	R	R	R	R	I	S	S	S	S	R	S	S	S	R	
15	*Burns	R	R	R	R	R	R	S	S	S	I	I	S	R	R	S	R	
16	Urine	R	R	R	R	R	S	S	S	S	S	I	S	S	S	S	R	
17	Burns	R	R	R	R	R	R	S	S	S	I	S	S	R	R	S	R	
18	Burns	R	R	R	R	R	S	S	S	S	I	S	R	S	R	S	R	
19	Urine	R	R	R	R	R	S	S	S	S	S	S	S	S	S	S	R	
20	Burns	R	R	R	R	R	R	S	I	R	R	R	S	S	S	S	R	
21	Urine	R	R	S	S	R	R	S	S	S	S	S	S	S	S	S	R	
22	*Sputum	R	R	R	R	R	R	S	S	S	S	S	S	S	R	S	R	
23	*Urine	R	R	R	R	S	R	S	S	R	S	R	S	S	S	S	R	
24	*Burns	R	R	R	R	R	R	S	S	S	S	S	S	S	R	S	R	
25	*Blood	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	PDR
26	*Burns	R	R	R	R	R	R	R	R	R	R	R	R	S	R	S	R	
27	*Blood	R	R	R	R	R	R	R	R	R	R	R	R	S	R	R	R	
28	*Blood	R	R	R	R	R	R	R	R	R	R	R	R	R	S	R	R	XDR
29	*Burns	R	R	R	R	R	R	R	R	R	R	R	S	S	R	R	R	
30	*Urine	R	R	R	R	S	R	S	S	S	S	S	S	S	I	R	R	
31	Urine	R	R	R	R	R	R	S	S	S	S	S	S	R	I	R	R	MDR
32	Urine	R	R	R	R	R	R	S	S	S	S	S	I	S	R	S	R	

AX: Amoxicillin 25 µg, TIC: Ticarcillin 75 µg , AMC: Amoxi/Clavulanic acid 30µg,CTX: Cefotaxime 30 µg, CRO: Ceftriaxone 30 µg, CAZ: Ceftazidime 30 µg, IMP: Imipenem 10 µg, MEM: Meropenem 10 µg, CN: Gentamicin: 10 µg, AK: Amikacin 30 µg, TM:Tobramicine 10 µg , TE: Tetracycline 30 µg, DO: Doxycycline 30 µg CIP: Ciprofloxacin 5 µg, , C: Chloramphenicol 30 µg, F: Nitrofurantoin 300 µg. R: Resistances , I: Intermediate , S: Sensitive, MDR: Multidrug resistance, XDR :Extensive drug resistance, PDR: Pandrug resistance, ESBL: Extended spectrum β-lactamase. *: ESBL.

antimicrobial agents including Imipenem, Meropenem, 3rd generation cephalosporins and others (Moland et al., 2003; Poirel et al., 2004; Essack et al., 2009). Padilla et al. (2010) reported that resistance mechanisms such as efflux pump can contribute to virulence of *Klebsiella*, and there was positive relationship between production of ESBL with virulence factors (Wiskur et al., 2008). As conclusions *fimH*-1, *ycfM* and *entB* genes were commonly found in all isolates, they seem to be at the basis of classic pathogenicity of *K. pneumoniae*. The most common virulence genes were observed in clinical sources of burns and blood infections. *K. pneumoniae* became highly resistant to antibiotics especially to 3rd generation cephalosporins. There was positive relationship between resistance to antibiotics and prevalence of virulence factors in *K. pneumoniae.*

RECOMMENDATIONS

Study the prevalence of another virulence gene in *k. pneumoniae* such as *kpn, traT and iroN*. And study the prevalence of virulence genes in another bacterium like *serratia* and *streptococcus pneumoniae.*

Conflict of Interests

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