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Determination of multi-drug resistant Klebsiella pneumoniae isolated from UTI patients in Wasit province, Iraq

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Abstract---The noticeable increase in the occurrence of multidrug-resistant *Klebsiella pneumoniae* strains separated from different hospitals in Wasit Province-Iraq demonstrates the limitation of antibiotics used for bacterial eradication. The aim of the present study is to detect the virulence genes in some *K. pneumoniae* isolates that collected from different hospitals in Wasit Province-Iraq. A total of 525 clinical samples were used to isolate 77 *K. pneumoniae* strains from clinical specimens throw fife months. They were identified by microbiological method as *K. pneumoniae*. The antimicrobial susceptibility of *K. pneumoniae* isolates was determined. The existence of virulence genes (AcrAB and tolC) were performed by PCR. The multidrug-resistant isolates showed resistance against Gentamicin (3.89%), Tobramycin (15.58%), and Amikacin (12.98%), which belongs to aminoglycoside antibiotics. Morever, βeta-Lactam antibiotics include Penicillins: Ampicillin (100%), Augmentin (19.48%), Carbapenems class including: Polymyxin-B (2.59%), and Imipenem (19.48%), Tetracyclins represented by Tetracycline (27.27%), Quinolones class including Ciprofloxacin (19.48%), Levofloxacin (14.28%), and Norfloxacin (14.28%), Macrolidies class including Azithromycin (57.14%),Amphenicols class including Chloramphenicol (1.29%),Nitrofurans class including Nitrofurantion (57.14%), finally Co-Trimoxazole class including Trimethoprim (33.76%). Molecular diversity between *K. pneumoniae* isolates was determined using

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Multiplex PCR technique. Results showed that out of 77 isolates there was 60 isolates (77.9%) positive to acrAB, 60 isolates (77.9%) to tolC.

Keywords---multi-drug resistant, *Klebsiella pneumonia*, UTI patient.

Introduction

Klebsiella pneumoniae, a member of the family Enterobacteriaceae, is a part of the flora and it is isolated as the causative agent in hard infections. It is an microorganism that causes severe diseases such as pneumonia, septicemia, bacteremia, wound infections and purulent abscesses at different sites in human. *K. pneumonia* is widely distributed in the urinary, respiratory, and gastrointestinal tracts of heal- thy people, most *K. pneumonia* are hospital associated with a high fatality rate if incorrectly treated (Martin and Bachman, 2018). *Klebsiella pneumonia* is chiefly responsible for intra-abdominal infections and hospital-acquired urinary tract infections, also *K. pneumonia*e has been described as the cause of highly persistent community-acquired infections, this bacterium is among the most commonly encountered nosocomial pathogens since the mid-1980s (Surgers *et al.*, 2016; Vasaikar *et al.*, 2017). The mechanisms resistance in *K. pneumonia* to various antibiotics classes included; production of antibiotic- inactivating enzymes, a variation of antibiotic target sites, changing of cell membrane permeability, efflux pump systems, and modification of metabolic pathways (Verma *et al.,* 2015). Certain mechanisms are either intrinsically encoded or acquired by the acquisition of resistance genes (Bialek-Davenet *et al.,* 2014). Among these mechanisms, efflux pump systems and enzymatic degradation play a significant role in increasing multidrug resistance (MDR) *K. pneumoniae*. *K. pneumonia* was observed high develop antibiotic resistance due to the production of new enzymes that break down antimicrobials more easily than most bacteria (Venkatachalam *et al.,* 2014).

Materials and Methods

Samples collection

This study was carried out in Al-Haj Jalal hospital in Wasit Province-Iraq. A total of 525 specimens from patients, clinically diagnosed as having UTI, during the period from 1st October to 30th February, 2021. Of these patients 442 were females (aged from 25 days to 80 years) and 83 were males (aged from 1 month to 80 years). All urine samples were collected in sterile screw capped test tubes.

Isolation and identification

Clinical samples were cultured onto MacConkey agar and Blood agar plates, incubated for 18-24 h at 37°C. All lactose-fermenting isolates were tested by morphologic characteristics according to MacFaddin, (2000).The collected isolates were identified biochemically according to Forbes *et al.*, (2002) and methods described by Macfaddin (2000). Confirmation of *K. pneumonia* was conducted using API20E system. The test was done according to the manufacturer's company instructions (BioMeriux/ France).

Maintenance of the isolates

Bacterial isolates were stored for long time in BHI broth containing 20% glycerol at -20°C (deep freezing), (Vandepitte *et al.,* 2003; WHO, 2003).

Antimicrobial susceptibility testing

All isolates were subjected to susceptibility test by modified disk-diffusion method (CLSI, 2021). Antibiotic resistance was determined using 15 antibiotic discs listed in table (2-1) according to the guidelines recommended by CLSI (2021), corresponding to the drugs considered in routine testing and reporting on *Enterobacteriaceae.* Uropathogenic *K. pneumoniae* isolates were prepared for sensitivity test by growing the picked colonies on TSA plates. The plates were incubated at 37˚C for 18-24 hr. Inoculums from the pure culture plate were prepared; a loopful of the growth was suspended in a tube of normal saline, the turbidity of the suspension was adjusted to equate that of 0.5 McFarland standards (1.5 x 10^8 CFU/ml). Muller Hinton agar plates were inoculated by dipping a sterile swab into the inoculums. The swab was streaked all over the surface of the medium several times. Antibiotic disks were applied to each plate by a sterile forceps. The plates were placed in an incubator at 37˚C for 18 hours. The diameter of each zone (including the diameter of the disk) were measured with ruler, and recorded in mm. The results then interpreted according to CLSI (2021) (Table 2-1).

Table 2-1
Inhibition zone diameter of antibiotics disk used in this study according to CLSI (2021)

Antibiotics (Symbol)	Inhibition zone diameter (mm)		
	S	I	R
Ampicillin (AMP)	≥17	14-16	≤ 13
Amikacin (AK)	≥17	15-16	≤ 14
Amoxicillin-clavulanic acid (AUG)	≥18	14-17	≤ 13
Azithromycin (AZM)	≥13	_____	≤ 12
Chloramphenicol (C)	≥18	13-17	≤ 12
Ciprofloxacin (CIP)	≥26	22-25	≤ 21
Imipenem (IMP)	≥23	20-22	≤ 19
Levofloxacin (LE)	≥ 21	17-20	≤ 16
Gentamicin (GEN)	≥ 15	13-14	≤ 12
Tetracycline (TE)	≥ 15	12-14	≤ 11
Polymyxin (PB)	_____	_____	_____
Trimethoprim (TR)	≥16	11-15	≤10
Norfloxacin (NX)	≥17	13-16	≤12
Nitrofurantion (NIT)	≥17	15-16	≤14
Tobramycin (TOB)	≥15	13-14	≤12

Molecular study

DNA was extracted from 77 *K. pneumonia* clinical isolates using a commercial purification system Easy Pure® Bacteria Genomic DNA Kit**.** The extraction of genomic DNA was performed according to the manufacturing of the company. Aseptically, PCR reaction mix was prepared using Taq Ready master mix Kit according to the manufacturer's instructions for a final reaction volume of 50 µl with 7µl of DNA extract. Multiplex PCR of each primer was performed with Taq Green Master Mix PCR Kit. The sequence of oligonucleotide forward and reverse primers which were used to detect *acrAB* and *tolC* are listed in table (2-2).

Table 2-2
Primer sequence of *Klebsiella pneumonia*

Gene	Primer sequence (5′- 3′)	Size of product (bp)	Reference
acrAB	F. CGCCTGAAGCAGGAGCTA R. GTGATTGAGCCGGTGGTC	152 bp	Designed in this study
tolC	F. GTCCATTACTGCCTCAGCTTG R. CTGATTGCTCTGATCCGTGA	200 bp	Designed in this study

Detection of antibiotic resistance genes *acrAB* and *tolc*

Reaction mix 50 µl consisted of 25 µl of 1X PCR Master Mix, 7.5 µl of resistance genes (3.75 µl from *acrAB*,, and 3.75 µl from *tolc)* of each primer, and 7 µl (10–100 ng) of template DNA and then complete the volume into 50 µl by nuclease-free water. DNA amplification was carried out with the following thermal cycling: an initial denaturation of DNA at 95 °C for 15 min. was followed by 35 cycles of amplification (95 °C for 40 sec., 52 °C for 30 sec. and 72 °C for 45 sec.), ending with a final extension at 72 °C for 5 min. and soak at 4°C for 5 min. table (2-3).

Table 2-3
Thermal cycling parameters of multiplex PCR reaction after optimization

Steps	Temperature (°C)	Time	No. of Cycles
Initial denaturation	95°	15 min	1
Denaturation	95°	40 sec	35
Annealing	52°	30 sec	
Extension	72°	45 sec	
Final extension	72°	5 min	1
Soak	4	5 min	1

Electrophoresis results were identified using UV-Transilluminator system. The DNA bands were measured according to the ladder DNA. The positive results were distinguished when there was DNA band equal to the target product size and then photographed using a camera.

Statistical Analysis

The data results of this study were analysed by using Graph Pad Prism 8 software and Microsoft Excel 2013 for each biological replicate. The level of probability at P values below of ≤ 0.05 that used to identify a significant difference.

Results and Discuss

Of 525 patients, clinically diagnosed as having UTI, only 233 (44.38%) were positive for bacterial culture (only one specimen was selected per patient).This result was consistent with other Iraqi researchers. In a study conducted by Alsamarai and Ali (2016) in Tikrit reported that 234 out of 563 (41.6%) gave positive cultures. In addition, Al-Jemely (2017) in Baquba 135 urine samples only 110 Samples gave bacterial growth 81.4 %.Also, Bachay (2018) in Wasit, 278 out of 774 specimens (35.9%) were positive for bacterial culture. Identification of *K. pneumonia* isolates based on morphological characteristics of the colonies on MacConkey agar,and blood agar. *Klebsiella pneumonia* isolates appeared large, mucoid and pink on MacConkey agar due to lactose fermenting while on blood agar they appeared white, large, mucoid colonies without hemolysis (Liu and Guo, 2019).

The biochemical tests were used for identification of bacterial isolates. Table (3-1) showed that All isolates of *K. pneumonia* gave positive catalase test indicated by bubbles formation of O2 (Forbes *et al.*, 2014).While the results of IMViC differentiate them from other lactose fermenter genera showed negative result for indole (Collee *et al.*, 1996).a positive result for citrate utilization test, Utilization of citrate is important physiological test *Klebsiella* showed positive reactions for citrate (Macfaddin, 2000). All samples gave positive result for urease production due to ability to produce the enzyme urease, these enzymes converted the color from yellow to pink (Brooks *et al.*, 2007).

Table 3-1
Biochemical tests used for confirming the identification of *K. pneumonia* isolate

Tests	*K. pneumonia*
Vogesproskauer	+
Indol test	-
Citrate uti-lization test	+
Methy red	-
TSI	A/A/G+/H2S-
Oxidase	-
Catalase	+
Urease test	+

(+) : Positive result ; (-) : Negative result ; A : Acid ; G+: Gas production; H2S- : No black sediment

API 20E System Identification of *Klebsiella pneumoniae*

All isolates were confirmed by using API 20E system as shown in figure (3-1)

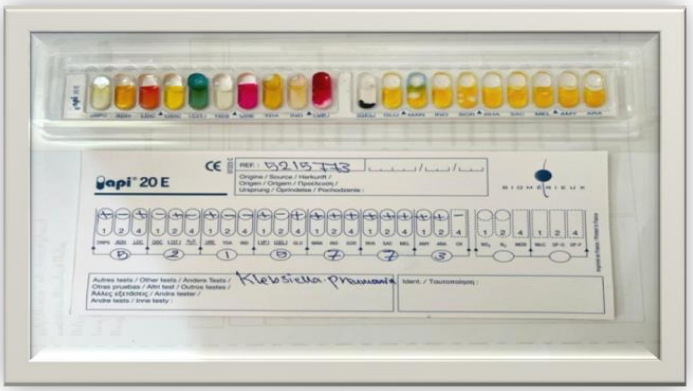

Figure 3-1. API 20E system for *K. pneumoniae* characterization

Of 233 patients with positive bacterial culture, isolated *E. coli* from79 (33.05%), followed by *K. pneumonia* 77(32.21%), *Staphylococcu aureus* 65 (27.19%), *Citrobacter* 6 (2.51%), *Proteus* spp. 5 (2.09%), *Enterobacter* 3 (1.25%), and *P. aeruginosa* 3 (1.25%), and *Streptococcus Pneumoniae* 1 (0.41%) distribution of isolates was depicted in Table (3-2). This study's results agreed with other local studies such as Essa *et al.* (2016) performed a study in Baghdad and Arbil, on pregnant women, isolated *E. coli* from 49.1% followed by *Acinetobacter baumannii* (21.3%), *K.pneumoniae* (13%), *P. mirabilis* (11.1%), and *P.aeruginosa* (5.6%). Bachay (2018) performed a study in Wasiton women, isolated *E. coli* from 67.20%, followed by *K.pneumoniae* (15.90%), *Proteus* spp. (6.70%), *P. aeruginosa* (6.70%) and *Enterobacter* (3.36%). Among UTI caused by *K. pneumoniae* females had a higher frequency than males as shown in Table (3-2).In term of age and gender, the highest frequency of UTI was among young women (15-40 years) as shown in Table (3-3). This agreed with other studies, Bachay (2018) found that the highest frequency of UTI (51.7%) was occurred in women that aged 15-40 years in Iraq. Iranpour *et al.* (2015) clarified that 50% of patients with UTI aged 15-45 years In Iran.

Table 3-2
Positive bacterial culture distribution among 233 patients with UTI

Type of Bacteria	No. Bacteria
Escherichia coli	79
Klebsiellapneumoniae	77
Staphylococcuaureus	65
Citrobacter	6
Proteus	5
Enterobacter	3
Pseudomonas aeruginosa	3
Streptococcus Pneumoniae	1
Total	239

Table 3-3
Gender and age distribution of *K. pneumonia* isolates from patients with UTI

Patients' gender	Patients' age group	No. of *K. pneumoniae* (%)
Females	1month-14 years	15 (19.5%)
	15-40 years	(50.6%) 39
	>40 years	6 (7.7%)
Total		60 (77.8%)
Males	1month-14 years	8(10.4%)
	15-40 years	7 (9.1%)
	>40 years	2 (2.6%)
Total		17 (22.1%)

Antimicrobial resistance of *K. pneumonia* isolates

Antimicrobial susceptibility test towards fifteen antibiotics was determined using agar disc diffusion test (Kirby-Bauer method) according to the Clinical Laboratory Standards Institute (CLSI) guidelines (2021). The isolates showed a variable levels of resistance to antibiotics used in this study, the isolates showed resistance to Gentamicin (3.89%), Tobramycin (15.58%), and Amikacin (12.98%), which belongs to aminoglycoside antibiotics. Morever, βeta-Lactam antibiotics include Penicillins: Ampicillin (100%), Augmentin (19.48%), Carbapenems class including: Polymyxin-B (2.59%), and Imipenem (19.48%), Tetracyclins represented by Tetracycline (27.27%), Quinolones class including Ciprofloxacin (19.48%), Levofloxacin (14.28%), and Norfloxacin (14.28%), Macrolidies class including Azithromycin (57.14%), Amphenicols class including Chloramphenicol (1.29%), Nitrofurans class including Nitrofurantion (57.14%),finally CTrimoxazole class including Trimethoprim (33.76%),The overall susceptibility patterns of isolates are displayed in Figure (3-2), table (3-4).

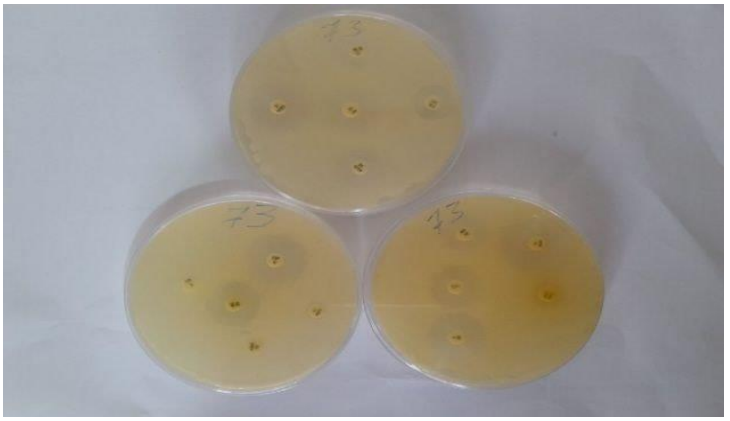

Figure 3-2. Antibiotic sensitivity test of *Klebsiella pneumoniae* isolate no.(73)

Table 3-4
Antimicrobial resistance of *Klebsiella pneumoniae*

Antibiotics	Resistance Percentage	Intermediate Percentage	Sensitive Percentage
	R (%)	I (%)	S (%)
Amoxicillin/ clavulanic acid (AUG)	15 (19.48)	7 (9.09)	55(71.42)
Ampicillin (AMP)	100 (100.0)	0	0
Azithromycin (AZM)	44 (57.14)	0	33 (42.85)
Amikacin (AK)	10 (12.98)	9 (11.68)	58 (75.32)
Ciprofloxacin (CIP)	15 (19.48)	21 (27.27)	41 (53.24)
Chloramphenicol (C)	1 (1.29)	7 (9.09)	69 (89.61)
Gentamicin (GEN)	3 (3.89)	7 (9.09)	67 (87.01)
Imipenem (IMP)	15 (19.48)	28 (36.36)	34 (44.15)
Levofloxacin (LE)	11 (14.28)	15	51 (66.23)
Norfloxacin (NX)	11 (14.28)	5 (6.49)	61 (79.22)
Nitrofurantion (NIT)	44 (57.14)	15 (19.48)	18 (23.37)
Tetracycline (TE)	21 (27.27)	6 (7.79)	50 (64.93)
Trimethoprim (TR)	26 (33.76)	6 (7.79)	45 (58.44)
Tobramycin (TOB)	12 (15.58)	16 (20.77)	49 (63.63)
Polymyxin (PB)	2 (2.59)	0	75 (97.40)

R: resistant; I: intermediate; S: sensitive

The emergence of resistant *K. pneumonia* bacteria is considered as an evidence of development of resistance, due to the possess mechanisms of resistance to carbapenems include production of lactamases and mutations that alter the expression and/or function of porins and PBPs (Walsh, 2010; Bleriot *et al.*, 2020). Combinations of these mechanisms can cause high levels of resistance to carbapenems in *K. pneumonia* (Suay-García and Pérez-Gracia, 2019; Ugakli and Dogan, 2020). It is very important for public healthcare departments to monitor and report the changes in antimicrobial-resistant isolates (Effah *et al.*, 2020). In some local study such as : Al-Mauwasi (2018) in Baghdad illustrated that the resistance rate of *K. pneumonia* clinical isolates was 38.57% resistant to Piperacillin, 42.85% for Impenem, 45.71% for Ciprofloxacin, 55.71% for Tetracycline, 55.71% for Gentamycin, 62.85% for Amikacin, 74.28% to Augmentin, and 81.42% for Cefotaxime.

Al-Hasnawi(2020)inAl-Najaf illustrated that the resistance rate of *K. pneumonia* clinical isolates was 98.7% resistant to penicillins, 89.1% resistant to amoxicillin-clavulanic acid, the rates resistance to cefotaxime, ceftazidime, ceftriaxone, and cefepime were 89.8%, 81.6%, 83.7%, and 71.4%, respectively. In addition, cefoxitin (68%), imipenem and meropenem 7.5% and 12.2%, respectively; and resistant to of loxacin, levofloxacin and Norfloxacin was 50.3%, 38.1%, and 45%, respectively; and (2%) resistant to colistin. In another recent local study done by Al-Rubyaie (2021)in Baghdad showed that the percentage of Impenem and Amikacin were about 16.0% **,**against *K. pneumonia* isolates, while the percentage of resistance to Piperacillin was 28.28%, the percentage of resistance to Ciprofloxacin was 36.0%, the percentage of resistance of Cefotaxime was 80.0%, followed by 100% to both of Augmentin and Tetracycline. A study by Nirwati *et*

al., (2019) in Indonesia showed that *K. pneumonia* isolates were showed resistance of rate 38.75% and 36.69%, for Ciprofloxacin and Amoxicillin-Clavulanic acid, respectively. and 100% sensitive to Amikacin. Another study done by Cepas *et al.* (2019) in Catalonia, showed that 40% of *K. pneumonia* strains were resistant to Amoxicillin-Clavulanic acid and Ciprofloxacin.

The relative variation in the patterns of resistance of *K. pneumonia* towards the antibiotics occurs due to many reasons like geographical differences, size of studied samples, site of infection, source of specimens, and predisposition patient, but it is generally seen from the general context of the antibiogram that the *K. pneumonia* were multidrug resistant pathogens and they have a relatively high level of resistance to Cephalosporins (Martínez and Baquero, 2002; Abdel-Rhman, 2020).Interestingly, the current finding revealed that *K. pneumonia* isolates showed resistance to antibiotics. *K. pneumonia* isolates are differentiated into 41 pattern (Table 3-5), the highest rate of multidrug resistance (MDR) was observed with pattern (1-7) in which these isolates were able to resist about (9→12) antibiotics. In pattern (1), isolates were able to resist twelve antibiotics which used in this study. The lowest MDR were noticed with pattern 38,41 which the total number of resisted antibiotic were only 2, 1 respectively.

A recent local study by Kareem *et al.* (2021) in Baghdad revealed the emergence of efflux pump-mediated drug resistance in MDR *K. pneumonia* bacteria. Another recent study in Balochistan for a total (107) clinical *K. pneumonia* isolates showed that all (107) isolates were MDR to minimum 6 and maximum 14 antibiotics out of 17 (Fatima *et al.*, 2021).However, the current results have shown consistent with other study conducted by Hussein (2018) which showed variable levels of resistance in urinary *K. pneumonia* isolates which have, in general, high rates of resistance to the most commonly used antimicrobial agents. It is necessary to follow proper infection control practices and physicians should be aware of the patients with such risk factors.

Table 3-5
Pattern of antibiotics resistance distribution among isolates of *K. pneumonia* according to number of antibiotic resist

Patterns	Total No.	Antibiotic resistance pattern	No. of antibiotic resist
1	1	AK, AMP, AUG,AZM,CIP,GEN,IPM,NIT,NX,TE,TR,TOB	12
2	1	AMP, AZM,C,CIP,GEN,LE,NIT,NX,TE,TOB,TR	11
3	1	AK, AMP,AZM,AUG,CIP,IPM,LE,TE,TR,TOB	10
4	1	AK,AMP,AZM,AUG, IPM,NIT,NX,TE, TOB	9
5	1	AMP,AZM,AUG, IPM,NIT,NX,TE, LE,CIP	9
6	1	AK,AMP,AZM,CIP,NIT,NX,TR, TOB,GEN	9
7	1	AMP,AZM,AUG, IPM,NIT,TOB,TE, TR,CIP	9
8	1	AMP,AZM,AUG, IPM,NIT,NX,LE, CIP	8
9	2	AMP,AZM,TE, LE,TR,CIP,NX	7
10	1	AK,AMP,CIP,IMP,NX,LE,TR	7
11	1	AMP,AZM,TE,TR,CIP,NIT	6

12	1	AMP,AZM,TE,TR,IPM,NIT	6
13	1	AMP,CIP,LE,IPM,NIT,NX	6
14	1	AMP,AZM,CIP,NIT,TR,TOB	6
15	1	AK,AMP,AUG,AZM, NIT,TOB	6
16	1	AMP, CIP, IPM,NIT,NX,LE	6
17	1	AK,AMP,AUG,AZM, NIT,TOB	6
18	3	AMP,AZM, NIT,TR,TE	5
19	2	AMP,AZM,TE,TR, AUG	5
20	1	AMP,NIT,TE,TR,LE	5
21	1	AMP,AZM,NIT,IMP,PB	5
22	1	AMP,AZM,NIT,IMP,TOB	5
23	1	AMP,AZM,NIT,CIP,TR	5
24	2	AK,AMP,AUG,AZM,TE	5
25	3	AMP,AZM,TE,TR	4
26	2	AMP,AZM,NIT,IMP	4
27	1	AK,AMP,AUG,AZM	4
28	1	AMP,NIT,TE,TR	4
29	1	AMP,AZM,PB,TR	4
30	1	AMP,AZM,NIT,TR	4
31	1	AMP,AUG,NIT	3
32	1	AMP,AUG,TR	3
33	1	AMP,AZM,LE	3
34	1	AMP,AZM,TR	3
35	1	AMP,AK,NIT	3
36	5	AMP,AZM,NIT	3
37	1	AMP,IPM,NIT	3
38	5	AMP,AZM	2
39	11	AMP,NIT	2
40	2	AMP,TOB	2
41	10	AMP	1

Genotyping of Antibiotics Resistance Genes of *Klebseilla pneumonia* Using Multiplex PCR

K. pneumonia isolates were typed genotypically by using Multiplex-PCR. Amplification of genes by multiplex PCR technique was done for 77 isolates of *K. pneumonia* to detect antibiotics resistance genes: acrAB and tolC. Results showed that out of 77 isolates there was 60 isolates (77.9%) positive to acrAB and 60 isolates (77.9%) to tolC as shown in figure (3-3). Ferreira1*et al.*,(2019)in Brazil reportedthe genes related with efflux pumps found were AcrAB (100%), tolC (96%). Mirzaie, and Ranjbar, (2021) in Tehranthe efflux pump genes including *AcrAB* and *tolC* were observed in (41%) and (33%) of the strains respectively.

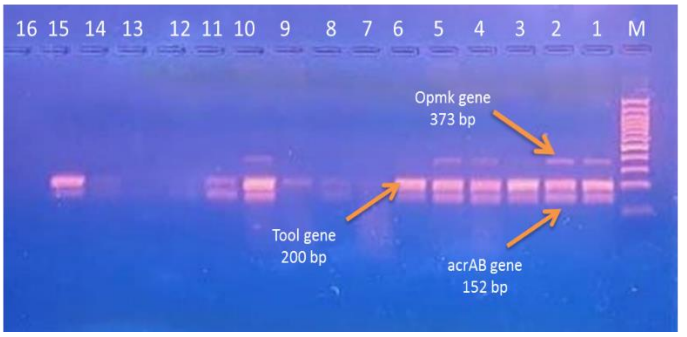

Figure 3-3. Agarose gel electrophoresis image that showed the multiplex PCR product of two genes *(tool and acrAB) of Klebsiella pneumoniae* isolates at 200 and 152 bp PCR product size respectively. The Lane (M): DNA marker (100-1500bp)

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