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ORIGINAL ARTICLE

Type VI secretion system (T6SS) in *Klebsiella pneumoniae*, relation to antibiotic resistance and biofilm formation

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ABSTRACT

Background and Objectives: The type VI secretion system (T6SS) was identified as a novel virulence factor in many Gram-negative bacteria. This study aimed to investigate the frequency of the T6SS genes in *Klebsiella pneumoniae*-causing different nosocomial infections, and to study the association between T6SS, antibiotic resistance, and biofilm formation in the isolated bacteria.

Materials and Methods: A total of fifty-six non-repetitive *K. pneumoniae* isolates were collected from different inpatients admitted at Sohag University Hospital from September 2022 to March 2023. Samples were cultured, colonies were identified, and antimicrobial sensitivity was done by VITEK® 2 Compact. Biofilm formation was checked using Congo red agar method. T6SS genes, and capsular serotypes were detected by PCR.

Results: Fifty-six *K. pneumoniae* isolates were obtained in culture. 38 isolates (67.86%) produced biofilm and 44 (78.57%) were positive for T6SS in PCR. There was a significant association between the presence of T6SS and resistance to the following antibiotics: meropenem, ciprofloxacin, and levofloxacin. All biofilm-forming bacteria had T6SS, with significant differences towards T6SS -positive bacteria. There was no significant association between T6SS, and the presence of certain capsular types.

Conclusion: The T6SS-positive *K. pneumoniae* has greater antibiotic resistance, and biofilm-forming ability which is considered a potential pathogenicity of this emerging gene cluster.

Keywords: Type VI secretion system; Biofilm; Antibiotic resistance; *Klebsiella pneumoniae*

INTRODUCTION

Klebsiella pneumoniae is a Gram-negative bacterium that belongs to the family Enterobacteriaceae. It causes a wide range of infections including pneumonia, urinary tract infections, bacteremia, and liver abscesses. These infections have high rates of antibiotic resistance and virulence factors all over the world (1).

One of these virulence factors is the protein secretion system. It is a characteristic component on the cell surface of Gram-negative bacteria. Many Gram-negative bacteria encode a molecular machine called the type VI secretion system (T6SS), which is

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an important microbial communication factor within human hosts, and the surrounding environment (2).

The bacterial Type VI Secretion System (T6SS) is a membrane-attached tube resembling contractile phage physically, and mechanistically (3). Different substances like antibacterial, and anti-eukaryotic effectors are injected through this system by a rapid conformational shift in the structural frame of a sheath protein complex (4).

Functioning components of T6SS are Hemolysin-coregulated protein (HCP) secreted into the extracellular environment (5), and valine-glycine repeat protein (VgrG) (6). Since HCP and VgrG fall into the extracellular environment by activating the system, they also act as molecular detectors of a functional T6SS (7). Also, T6SS has an intracellular portion, the intracellular multiplication protein F (ICMF) family which is a T6SS inner membrane protein that has ATPase or proton motive forces that are commonly used to energize secretion apparatus assembly, and/or substrate transfer (8).

The role of T6SS of *K. pneumoniae* in drug resistance, and biofilm formation is still unclear. Here we characterized the T6SS in clinical *K. pneumoniae* isolates collected from inpatients in Sohag University Hospital to analyze the potential association between T6SS, and antibiotic resistance and biofilm formation.

MATERIALS AND METHODS

This cross-sectional study was conducted at the Departments of Clinical Pathology and Medical Microbiology and Immunology, Faculty of Medicine, Sohag University from September 2022 to March 2023 after obtaining ethical approval and institutional review board (IRB) number: Soh-Med-22-08-23.

Sample collection and identification. Different hundred clinical samples (blood, urine, pus, sputum, aural swap, ascetic fluid, throat swap, stool, bronchial aspirate, and nasopharyngeal swab) from patients admitted to the hospital in different departments were collected under sterile conditions, and sent to the laboratory, cultured on routine culture media, of them 56 isolates were identified as *K. pneumoniae* and tested against antibiotics using VITEK® 2 Compact (bioMerieux). Bacteria other than *Kebsiella* were excluded from the study. Bacteria were preserved on trypticase soya broth with 20% glycerol, and stored at -60°C for further use.

Biofilm detection. Biofilm was detected by Congo red agar method (9). The isolated bacterial strains were inoculated into Congo red agar plates (CRA) and incubated for 24-48 hours at 37°C. CRA preparation was as follows: Brain heart infusion (BHI) broth (37 g/L), agar (10 g/L), (5%) sucrose, and Congo red stain (0.8 g/L). Congo red (Oxoid) is an aqueous solution that is autoclaved at 121°C for 15 minutes alone, and added when the agar cooled down to 55°C. Isolates were considered positive when black colonies with a dry crystalline appearance. Colonies with pink color were recorded as non-biofilm producers.

DNA extraction and PCR. DNA was extracted from freshly subcultured bacteria according to the method of Tarchouna et al. (10), and extracted DNA was stored at -20 for subsequent use. PCR was used to detect genes of capsular typing (k1, k2) and type 6 secretion system genes (HCP, VgrG, and ICMF). Sequences of primers, cycling condition and amplicon size are summarized in Table 1. Each PCR reaction was adjusted to a total volume of 25 µL using the following reaction mixture: 12.5 µL of cosmo red PCR Master Mix [2x] (Willowfort, Uk), 1 µL of forward primer, 1 µL of reverse primer, 3 µl of template DNA then the reaction was adjusted to 25 µL with nuclease-free water. Negative control tubes were also included without a DNA template. After amplification, 10 µLof the PCR mixture was analyzed by agarose gel electrophoresis (2% agarose in Tris acetate-EDTA stained with ethidium bromide). The Gene Ruler 50 bp DNA ladder (Invitrogen, Thermo Fisher) was used as a DNA size marker visualization of bands was done using a DNA documentation system.

Statistical analysis. Data were analyzed using STATA version 17.0 (Stata Statistical Software: Release 17.0 College Station, TX: Stata Corp LP). Quantitative data were represented as mean, standard deviation, median, and range. Qualitative data were presented as numbers and percentages, and compared using either the Chi-square test or the Fisher exact test. Graphs were produced by using the Excel program. The P-value was considered significant if it was less than 0.05.

Table 1. Primers sequences of different *Klebsiella pneumoniae* genes with their cyclic conditions in conventional PCR

	Sequence Gene	Cycling condition	Amplicon size (bp)	Reference
K1	F: GTAGGTATTGCAAGCCATGC R: GCCCAGGTTAATGAATCCGT	95°C/5 min; (95°C/1 min, 45°C/30sec, 72°C/45sec) X30; 72°C/5min	1024	(11)
K2	F: CTGGAGCCATTTGAATTCGGTG R: CTTCCCTAGCACTGGCTTAAGT	95°C/5 min; (95°C/1 min, 45°C/30sec, 72°C/45sec) X30; 72°C/5min	641	(11)
HCP	F: TCCCGACCGATAACAACAACACC R: GATGTCGTGCATCAGGGGAT	95°C/5 min; (95°C/1 min, 42°C/30sec, 72°C/45sec) X30; 72°C/5min	242	(12)
VgrG	F: TGAGCGTGTTTGTGCGAAAG R: TGACGCCCGTAATATCCTGC	95°C/5 min; (95°C/1 min, 42°C/30sec, 72°C/45sec) X30; 72°C/5min	259	(12)
ICM	F: GACCGCTTACGCTTACGGACAACTG R: CACTCAGCACCCAGTCCATT	95°C/5 min; (95°C/1 min, 44°C/30sec, 72°C/45sec) X30; 72°C/5min	495	(12)

RESULTS

Sixty-five positive *K. pneumoniae* were collected from patients admitted to different departments their age mean was 30.16 ± 29.09, 32 (57.14%) of them were females and 24 (42.86%) were males as in (Table 1). Samples were collected from different departments, the most frequent department was Pediatric 22 (39.29%) followed by ENT, ICU, and surgical departments 6 (10.71%) as demonstrated in (Table 2).

Different types of samples were collected from admitted patients, 18 (32.14%) were from blood followed by 12 (21.43%) samples from pus of infected wounds, and 6 (10.71%) were from urine as in Fig. 1.

Samples were processed, cultured and then identified and tested for their antibiotic susceptibility by VITEK® 2 Compact. The results demonstrated that all isolates (100%) were resistant to ampicillin, ampicillin sulbactam, cefazolin, cefoxitin, ceftazidime and ceftriaxone. Fifty-four (96.43%) were resistant to piperacillin, and cefepime. The results of antibiotic susceptibility testing is shown Table 3.

Of the isolated bacteria, 6 (10.71%) were extended-spectrum beta-lactamase (ESBL) producers (Table 4).

ESBL producers were detected by VITEK, AST cards of VITEK examine ceftazidime and ceftriaxone resistance alone and with clavulanate to detect ESBL-positive bacteria.

Phenotypic detection of biofilm production was done by Congo red method, and revealed that 38 (67.86%) of the isolated bacteria were biofilm positive by this method. Molecular typing of *K. pneumoniae* using K1, and K2 capsular genes were done by PCR, 8 (14.29%) were k1 type, 4 (7.14%) were k2 type, and the rest of the bacteria were non-k1, non-k2 types (Table 5).

Molecular detection of 6 secretion system genes was done by PCR, 44 (78.57%) of the isolated *Klebsiella* were harboring genes of type 6 secretion system (T6SS) as in Figs. 2 and 3.

Table 2. Age and sex distribution of the studied population

Variable	Summary statistics
Age/year	
Mean ± SD	30.16 ± 29.09
Median (range)	22 (0.02:80)
Gender	
Females	32 (57.14%)
Males	24 (42.86%)
Departments	Number (%)
Pediatric	22 (39.29%)
ENT	6 (10.71%)
ICU	6 (10.71%)
Surgery	6 (10.71%)
Tropical Medicine	4 (7.14%)
Chest	2 (3.57%)
Gynecology	2 (3.57%)
NICU	2 (3.57%)
PICU	2 (3.57%)
Orthopedic	2 (3.57%)
Urology	2 (3.57%)

ENT: ear, nose, and throat; ICU: Intensive care unit; NICU: Neonatal intensive care unit; PICU: Pediatric intensive care unit.

There was a significant association between the presence of type 6 secretion system and resistance to meropenem, ciprofloxacin, and levofloxacin (Table 6).

The relation between the type 6 secretion system and biofilm formation in *K. pneumoniae* was studied and reviled all biofilm-producing bacteria were harboring the secretion system, with significant differences in

the Type 6 secretion-positive bacteria (Table 7).

There was no significant association between T6SS and presence of certain capsular types (Table 8).

Studying the clinical characteristics of patients and their relation to Type 6 secretion, there was a significant difference between the positive system, and the following underlying conditions (Nosocomial infection, Malignancy, and Peripheral vascular diseases), and age group from 2 to 50 years, their median age is 8 (Table 9).

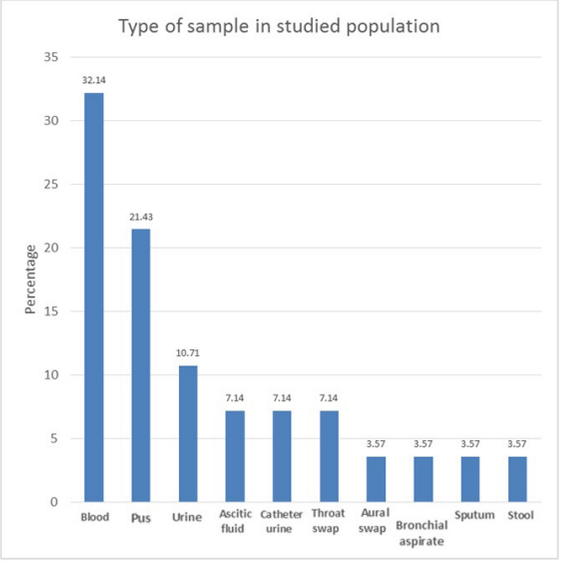

Fig. 1. Distribution of the studied population according to the type of sample

Table 4. Distribution of the studied bacteria according to ESBL production

ESBL	Number (%)
Negative	50 (89.29%)
Positive	6 (10.71%)

ESBL: Extended spectrum beta lactamase.

Table 5. Distribution of the studied bacteria according to the biofilm formation and capsular type

Biofilm formation	Number (%)
Negative	18 (32.14%)
Positive	38 (67.86%)
K1	
Negative	48 (85.71%)
Positive	8 (14.29%)
K2	
Negative	52 (92.86%)
Positive	4 (7.14%)

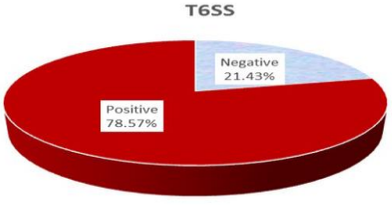

Fig. 2. Distribution of 6 secretion systems (T6SS) in isolated *Klebsiella pneumoniae*

Table 3. Antibiotic sensitivity of the isolated bacteria

Antibiotic	Sensitive	Intermediate	Resistance
Ampicillin	0	0	56 (100%)
Ampicillin sulbactam	0	0	56 (100%)
Piperacillin	2 (3.57%)	0	54 (96.43%)
Cefazolin	0	0	56 (100%)
Cefoxitin	0	0	56 (100%)
Ceftazidime	0	0	56 (100%)
Ceftriaxone	0	0	56 (100%)
Cefepime	2 (3.57%)	0	54 (96.43%)
Meropenem	8 (14.29%)	0	48 (85.71%)
Amikacin	24 (42.86%)	12 (21.43%)	20 (35.71%)
Gentamicin	18 (32.14%)	4 (7.14%)	34 (60.71%)
Tobramycin	6 (10.71%)	0	50 (89.29%)
Ciprofloxacin	18 (32.14%)	8 (14.29%)	30 (53.57%)
Levofloxacin	20 (35.71%)	6 (10.71%)	30 (53.57%)
Nitrofurantoin	12 (21.43%)	6 (10.71%)	38 (67.86%)
Trimethoprim-sulfamethoxazole	18 (32.14%)	0	38 (67.86%)

Table 6. Antimicrobial resistance of T6SS-positive, and T6SS-negative *K. pneumoniae* isolates

Antibiotic	Type 6 secretion			P value
	Negative N=12	Positive N=44	All N=56	
Ampicillin	12 (100%)	44 (100%)	56 (100%)	1.00
Ampicillin sulbactam	12 (100%)	44 (100%)	56 (100%)	1.00
Piperacillin	12 (100%)	42 (95.45%)	54 (96.43%)	1.00
Cefazolin	12 (100%)	44 (100%)	56 (100%)	1.00
Cefoxitin	12 (100%)	44 (100%)	56 (100%)	1.00
Ceftazidime	12 (100%)	44 (100%)	56 (100%)	1.00
Ceftriaxone	12 (100%)	44 (100%)	56 (100%)	1.00
Cefepime	12 (100%)	42 (95.45%)	54 (96.43%)	1.00
Meropenem	6 (50.00%)	42 (95.45%)	48 (85.71%)	0.001
Amikacin	4 (33.33%)	16 (36.36%)	20 (35.71%)	0.85
Gentamicin	6 (50.00%)	28 (63.64%)	34 (60.71%)	0.39
Tobramycin	12 (100%)	38 (86.36%)	50 (89.29%)	0.32
Ciprofloxacin	10 (83.33%)	20 (45.45%)	30 (53.57%)	0.02
Levofloxacin	10 (83.33%)	20 (45.45%)	30 (53.57%)	0.02
Nitrofurantoin	10 (83.33%)	28 (63.64%)	38 (67.86%)	0.30
Trimethoprim-sulfamethoxazole	10 (83.33%)	28 (63.64%)	38 (67.86%)	0.30

Table 7. Biofilm formation of T6SS-positive and T6SS-negative *K. pneumoniae* isolates

Biofilm formation	Type 6 secretion			P value
	Negative N=12	Positive N=44	All N=56	
Negative	8 (66.67%)	10 (22.73%)	18 (32.14%)	0.01
Positive	4 (33.33%)	34 (77.27%)	38 (67.86%)	

Table 8. Association between T6SS and different capsular types

			T6SS		Total	P value
			Positive	Negative		
K1	Negative	Count	36	12	48	0.11NS
		% within t6ss	81.8%	100.0%	85.7%	
	Positive	Count	8	0	8	
		% within t6ss	18.2%	0.0%	14.3%	
Total		Count	44	12	56	
		% within t6ss	100.0%	100.0%	100.0%	
			T6SS		Total	P value
			Positive	Negative		
K2	Negative	Count	40	12	52	0.27NS
		% within t6ss	99.9%	100.0%	92.9%	
	Positive	Count	4	0	4	
		% within t6ss	9.1%	0.0%	7.1%	
Total		Count	44	12	56	
		% within t6ss	100.0%	100.0%	100.0%	

*P < 0.05 (significant). **P < 0.01 (highly significant). ***P < 0.001 (very highly significant). NS: Non-significant p >0.05.

Table 9. Clinical characteristics of T6SS-positive, and T6SS-negative *K. pneumoniae* isolates

Clinical characteristics	Type 6 secretion			P value
	Negative N=12	Positive N=44	All N=56	
Age (median)	49 (45:65)	8 (2:50)	22.0 (2.25:57.5)	0.045*
Gender				
Female	4 (33.3%)	28 (63.6%)	16 (57.1%)	0.09
Male	8 (66.7%)	16 (36.4%)	12 (42.9%)	
Nosocomial infection	12 (100%)	24 (54.55%)	36 (64.29%)	0.005*
ICU admission	6 (50.00%)	26 (59.09%)	32 (57.14%)	0.57
Hypertension	4 (33.33%)	14 (31.82%)	18 (32.14%)	1.00
DM	2 (16.67%)	16 (36.36%)	18 (32.14%)	0.30
Malignancy	6 (50.00%)	2 (4.55%)	8 (14.29%)	0.001*
Cardiovascular disease	0	6 (13.64%)	6 (10.71%)	0.32
Cerebrovascular disease	4 (33.33%)	4 (9.09%)	8 (14.29%)	0.06
Chest problems	6 (50.00%)	24 (54.55%)	30 (53.57%)	0.78
GIT disease	2 (16.67%)	6 (13.64%)	8 (14.29%)	1.00
Renal disease	4 (33.33%)	4 (9.09%)	8 (14.29%)	0.06
Peripheral vascular diseases	4 (33.33%)	2 (4.55%)	6 (10.71%)	0.02*
Immunosuppression	2 (16.67%)	4 (9.09%)	6 (10.71%)	0.60

ICU: Intensive care unit. DM: Diabetes mellitus. GIT: Gastrointestinal disease

*P < 0.05 (significant). **P < 0.01 (highly significant). ***P < 0.001 (very highly significant). NS: Non-significant p >0.05.

There was no significant association between the type of certain sample, and the presence of T6SS.

DISCUSSION

The T6SS is structurally, and functionally like a bacteriophage tail encoded in many Gram-negative bacteria (13). This system helps bacteria to directly inject toxins, enzymes, and proteins to other bacteria or human cells. Its relation to drug, and biofilm formation still needs further studies.

In this cross-sectional study, 56 positive *K. pneumoniae* bacteria were collected from September 2022 to March 2023 from patients admitted to different departments at Sohag University Hospital. The age mean was 30.16 ± 29.09, females were 32 (57.14%), and males were 24 (42.86%).

Different types of samples were collected from admitted patients in different departments, 18 (32.14%) were from blood followed by 12 (21.43%) samples from pus of infected wounds, and 6 (10.71%) from urine.

From all isolated *K. pneumoniae*, 8 (14.29%) were k1 type, 4 (7.14%) were K2 type, and the rest of the bacteria were non-k1, nor k2 type near to the finding of Yin Zhang et al. (14). The presence of the three genes (HCP, ICMF, VIGH) was considered as positive T6SS, 44 (78.57%) of the isolated *Klebsiella* harboring genes of type 6 secretion system (T6SS) near to the results of Liao 2022 who detected T6SS in 179 (72.2%) clinical *Klebsiella pneumoniae*.

There was a significant association between the presence of this secretion system and resistance to the following antibiotics (Meropenem, Ciprofloxacin, and Levofloxacin) similar to the results of the study of Liao et al. (15).

Antibiotic resistance was more in T6SS-positive than T6SS-negative bacteria. This may be due to the acquisition of genes of resistance through conjugative plasmids that also carry genes of virulence. Frequency of antibiotic resistance was higher in T6SS-positive bacteria which is different from the results reported by Zhou et al. (12). This could be explained by differences in antibiotic usage between different localities, and stress on some antibiotics may be the cause of developing this resistance.

Next, we tried to study the association between the presence of T6SS and biofilm formation in the isolated bacteria. All biofilm-producing bacteria were harboring the secretion system, and a significant difference towards Type 6 secretion-positive bacteria

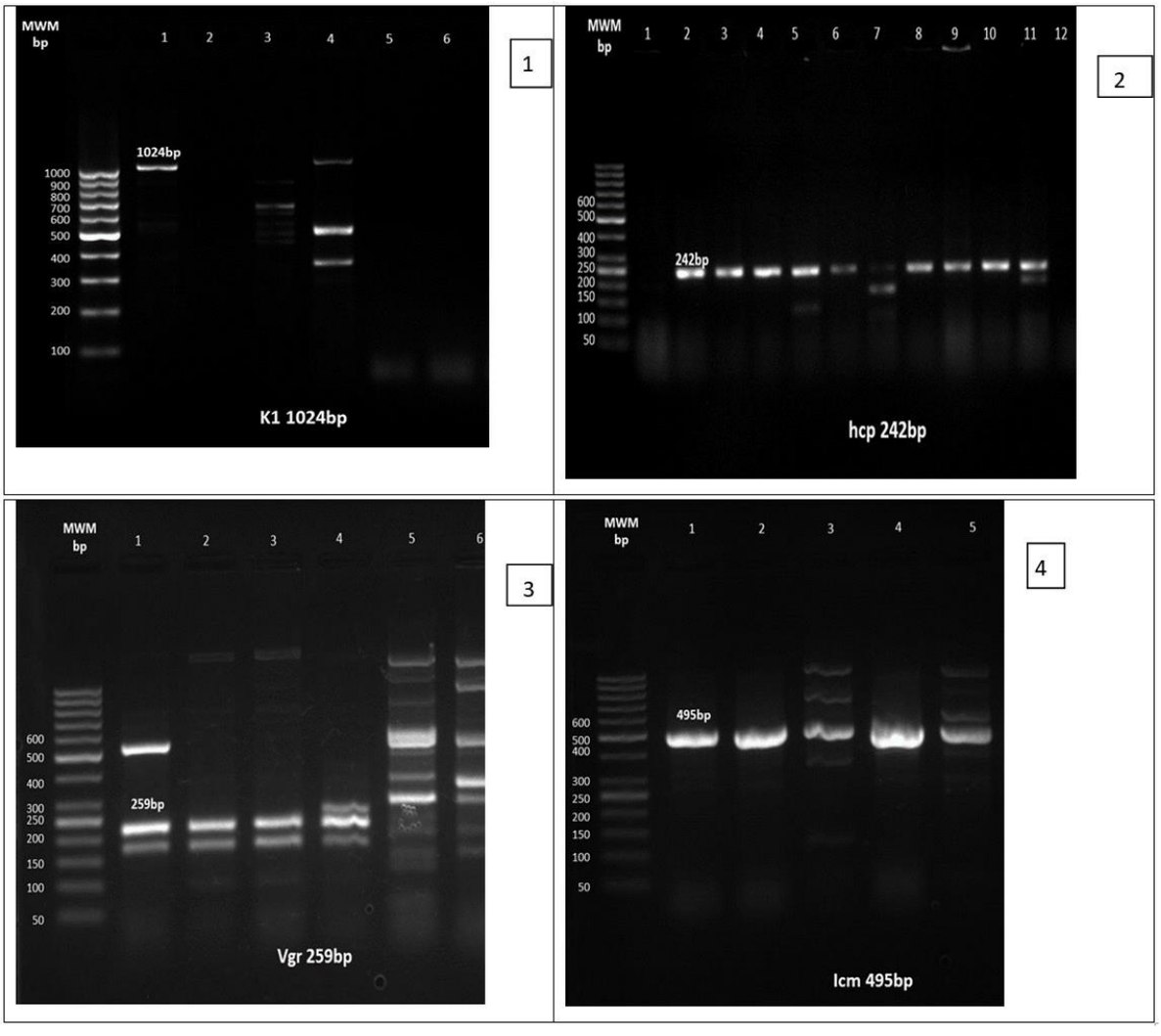

Fig. 3. 1- K1 gene with MW 1024bp detected in different bacterial isolates of *K. pneumoniae*; 2-hcp gene with MW 242 bp detected in different bacterial isolates of *K. pneumoniae*; 3- Vgr gene with MW 259 bp detected in different bacterial isolates of *K. pneumoniae*; 4- lcm gene with MW 495 bp detected in different bacterial isolates of *K. pneumoniae.*

was found, and could be explained by the exportation of different substances involved in biofilm formation through that secretion system. These results are similar to the results of Liao et al. (15) and Hsieh et al. (16).

Also, there was no significant association between T6SS, and capsular type (K1, K2) nor the different types of samples, that could be referred to a small number of our isolated bacteria. This result is dissimilar to that of Zhou et al. (12).

Concerning the clinical characteristics of patients and their relation to Type 6 secretion, there was a significant difference between the positive system, and the following underlying conditions (nosocomial infection, malignancy, and peripheral vascular diseases) dissimilar to Liao et al. (15) who found a significant association between T6SS, and diabetes and hypertension.

In conclusion, the prevalence of the type VI secretion system is high in our isolated *K. pneumoniae* in Sohag University Hospital. T6SS-positive strains show higher biofilm-forming activity, and high drug resistance acting as a virulence potential.

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