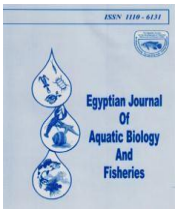
Egyptian Journal of Aquatic Biology & Fisheries
Zoology Department, Faculty of Science,
Ain Shams University, Cairo, Egypt.
ISSN 1110 – 6131
Vol. 26(6):1319 – 1328 (2022)
www.ejabf.journals.ekb.eg



Estimation of the Role of *Mrk* Genes in *Klebsiella pneumoniae* Isolated from River Waters and Clinical Isolates

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ARTICLE INFO

Article History:
Received: Nov. 30, 2022
Accepted: Dec. 17, 2022
Online: Dec. 30, 2022

Keywords:
Mrk genes,
Diyala River,
Skin infections,
Klebsiella pneumonie,
Swabs,
Adhesions

ABSTRACT

Klebsiella pneumoniae is a member of *Enterobacteriaceae*, which causes many infectious diseases including blood, lung, wound, burn, and urinary tract infections. It is found in sewage and river water as a coliform bacterium. This study aimed to find the prevalence and correlation among *Mrk* operon genes that express adhesion properties in both environmentally and clinically isolates. Twenty skin isolates of *K. pneumoniae* were collected from the wound and burn infections as swab samples. Fifteen samples of river water were obtained from the Diyala river and *K. pneumoniae* was isolated. The diagnosis was applied by characteristics on culture media and assured by VITEK 2 system with *16s rRNA* gene, then the DNA was extracted, and concentration and purity were measured for each isolate. PCR technique was performed to detect five genes of *Mrk* genes: *MrkA, Mrk B, Mrk C, Mrk D* and *Mrk F*. Findings illustrated that 10 of 15 water samples that contained *Klebsiella* besides the confirmed 20 clinical *K. pneumoniae* isolates express *Mrk* operon of the heterogeneous pattern. In clinical isolates, the percentages of *Mrk* genes (*Mrk A, Mrk B, Mrk C, Mrk D*, and *Mrk F*) were 75, 85, 80, 50 and 60%; while in environmental samples, they were 40, 60, 50, 20 and 40%, respectively, as the clinical isolates showed the largest occurrence of *Mrk* genes. A significant relationship was detected between the source of bacterial isolation and the prevalence of target genes (0.0039) at $P \leq 0.01$. The *Mrk* genes showed no correlation among them in water samples, except that recorded between *MrkB* and *MrkC*, which was 0.028 at $P \leq 0.01$. However, *Mrk* genes had a significant correlation at $P \leq 0.01$ and 0.05 among them in swab samples since they expressed more virulence persistence with high attachment in local skin infections, compared to river isolates.

INTRODUCTION

Klebsiella pneumoniae is a coliform bacterium, which causes serious illnesses in blood, lung, liver urinary tract and wounds; it is considered as opportunistically potent bacterium for both nosocomial and community acquired diseases **(Oliveira *et al.*, 2020)**. Adhesion phenomenon of *K. pneumoniae* is basically mediated by *Mrk* genes, coding the formation of globular proteins called type-3 pilus, which makes *K. pneumoniae* attach to host tissues and cells for initiating infection in addition to prosthetic valves, catheters, taps, pipes and tanks (**Chatterjee *et al.*, 2018)**. Type-3 pilus genes are very important to colonize, invade and persist *K. pneumoniae* and significant for biofilm formation on biotic and abiotic surfaces. *Mrk* gene cluster consists of five genes *(Mrk A, B, C, D* and *F)* **(Sonbol *et al.*, 2021)**.

Mrk genes are usually involved together with the antibiotic resistance as there is a significant association observed and documented with the persistence of *K. pneumoniae* for long time in

hospitals and the harsh environmental conditions, resulting in the difficulty of their elimination **(Mahmoud, 2020)**.

Correlation of *Mrk* genes with habitat was well-characterized in *K. pneumoniae* **(Scavuzzi *et al*., 2017)**. Many pathogenic or water isolates using pilus to initiate adhesion to host and environmental surfaces, facilitate bacterial interactions, which might promote resistance that subsequently result in increasing bacterial virulence and pathogenicity **(Eghbalpoor *et al*., 2017)**. *Klebsiella pneumoniae* harbor specific *Mrk* genes, which are mentioned to be affected phenotypically in their occurrence and expression as the clinical *K. pneumoniae* are capable to develop pathogenicity more specifically and fleetly than environmental isolates **(Caneiras *et al*., 2019)**. On the other hand, each one of the *Mrk* genes, encoded by specific plasmid was documented associated with the presence of the other genes of the same *Mrk* cluster **(Ragheb *et al*., 2020)**. Consequently, the current study aimed to evaluate the presence of adhesion *Mrk* genes in *K. pneumonia* clinical and river-isolates and determine the relationship with source of isolation among profile of *Mrk* genes.

MATERIALS AND METHODS

Samples collection

Twenty isolates of clinically pre-diagnosed *K. pneumoniae* collected from skin infection samples including wound and burn infections from three hospitals in September, 2022 of both genders of adult patients, with fifteen different samples of Diyala river water in clean, pre-sterilized containers.

Identification of Bacteria

The phenotypica investigation for *K. pneumoniae* isolates was initially determined via culture on selective medium (blood agar and MacConkey agar, Himedia; UK) for clinical and environmental samples by streaking and pouring methods, respectively. VITEK-2 compact system (Bio-Merieux- France) specific to Gram negative bacteria and used for detecting the species and genus of the identified bacterium including 64 biochemical and growth-related tests for confirming primary cultural features and morphological identification of isolated bactertia.

DNA Extraction

The extraction of DNA was achieved according to the kit leaflet of extracting whole DNA extraction kit-wizard; Promega-USA, and the purity with concentration of the DNA were both measured by nanodrop. Genotyping identification was performed by the *16s rRNA* house-keeping (HKG) gene for all *K. pneumoniae* isolates. The*16S rRNA* sequences forward primer was (5’ AGAGTTTGATCCTGGCTCAG 3’), and of the reverse primer (5’ TACGGTTACCTTGTT ACGACTT 3’) (Hasan, 2021). The PCR-mixture included 12.5 µl master mix (Promega - USA); with 3 µl template DNA, with 1.5 µl of each reverse and forward primers for the gene *16S rRNA* in addition to 6.5 µl nuclease free water; all were mixed using the vortex and the mixtures were all placed in the thermocycler, previously all the conditions of PCR stages were set by the software program. The temperature of completely initial-denaturation was 94 centigrade for 5 minutes for one cycle, followed by forty cycles each consisted of three PCR stages which were: denaturation via 94 centigrade for one minute, annealing via 60 centigrade for one minute and extension stage via 72 centigrade for one minute. The stage of final extension stage was applied at 72 centigrade for five minutes by single cycle only.

Molecular analysis of *Mrk* cluster genes

Target *Mrk* genes amplification was done by forty cycles of PCR as the same way of preparation of *16s rRNA* gene concentrations in PCR mixture mentioned previously. In current study, the design of all primers was achieved by the program geneious software, and the sequence of primers are shown in Table "1". Then electrophoresis was applied by 100 volt, for 45 minutes via using 1% concentration of agarose. Product of each amplified gene of *Mrk* cluster with their

specific forward or reverse primer were all sent to the company Macrogen in South Korea for DNA sequencing analyzed by using Sanger genetic method for sequencing besides performing blast hit was detected which resembles the precise position of amplified genes in the original genome size of *K. pneumonia* in comparsion with certain standard strain that is available in National Centre for Biotechnology Information (NCBI) for ensuring the target genes which belong to *K. pneumoniae*.

Table 1. Primers used for studied *Mrk* genes

Symbol of studied genes	Sequence of forward primer (5′to 3′)	Sequence of reverse primer (5′to 3′)	Size (bp)	Annealing temperature (centigrade)
Mrk A	GGC AGT T T TAT T T TCTGACGG	GCACT A A AC AG GATG AC GTAA	358	57. 4
Mrk B	GGCCTTCAT T T T TCTGATTGG	T TCAT T T T TAGT T T TGCGGCC	346	53. 4
Mrk C	GA ATTGT TGTAGCTGACCTGA	TCAACGCCTGAAAAACT ATGT	1155	58. 5
Mrk D	GA AC C CACATCGACAT TCATA	CAGCA A ACA ACA A AG GATAGC	628	57. 4
Mrk F	TA A ATAT TCTGCGCTCCATCC	GGATTGCCGA A A A ACACTATC	419	57. 4

STATISTICAL ANALYSIS

Statistical analysis was applied by SPSS statistical package of Social Sciences program (version 20.0 of windows, SPSS-Chicago- USA). The count and percentages were resembled as qualitative data. Chi square was used for testing relation between qualitatives of the same group of samples, while LSD was used for testing the differences between the two categories of samples (clinical and environmental). P-value which is $\leq$ 0.01 and 0.05 documented as statistically significant (SPSS, 2019).

RESULTS AND DISCUSSION

K. pneumonia Isolates Identification

The colonies of the bacteria *K. pneumoniae* were characterized by growth on the blood agar as viscous, circular, smooth, convex colonies having obvious edge, that ferment lactose but does not hemolyze blood in pre-diagnosed swab samples and in 10 of the total 15 collected water samples, then the diagnosis confirmed by using cards specific to Gram negative bacteria of VITEK 2 (ID GN 76) in addition to sequencing *16s rRNA* (housekeeping) gene according to NCBI database. The results of gel electrophoresis showed that the band of *16s rRNA* ranked 1500 bp position as obvious in figure 1(a); while figure 1(b) clarified electrophoresis bands in addition to the blast region which resembled the presice size of target gene (*16s rRNA* gene) of the original size of bacterial genome. PCR products which had positive gene occurrence for *16s rRNA* gene confirms the *K. pneumoniae* genome; via using NCBI data base for DNA sequence (Shanker, *et al*. 2018). Besides, the molecular diagnosis by using 16s *rRNA* gene was performed by many researchers (Al-Musawi, 2012; Hamed and Hasoon, 2019).

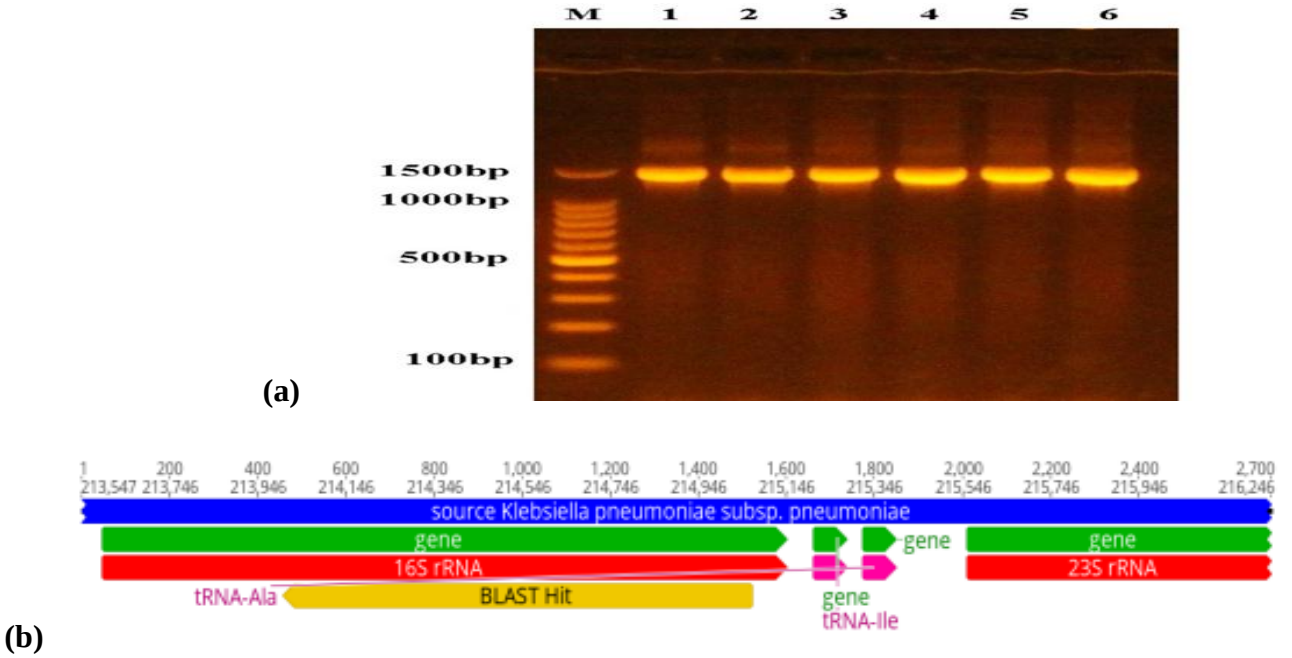

Figure 1. (a) Agarose electrophoresis for PCR bacterial product at 1500 bp for*16s rRNA* in 1% agarose in 100Volt for 45 minutes (b) Blast hit for *16s rRNA* gene regarding same sequence of NCBI strains of *K. pneumoniae.*

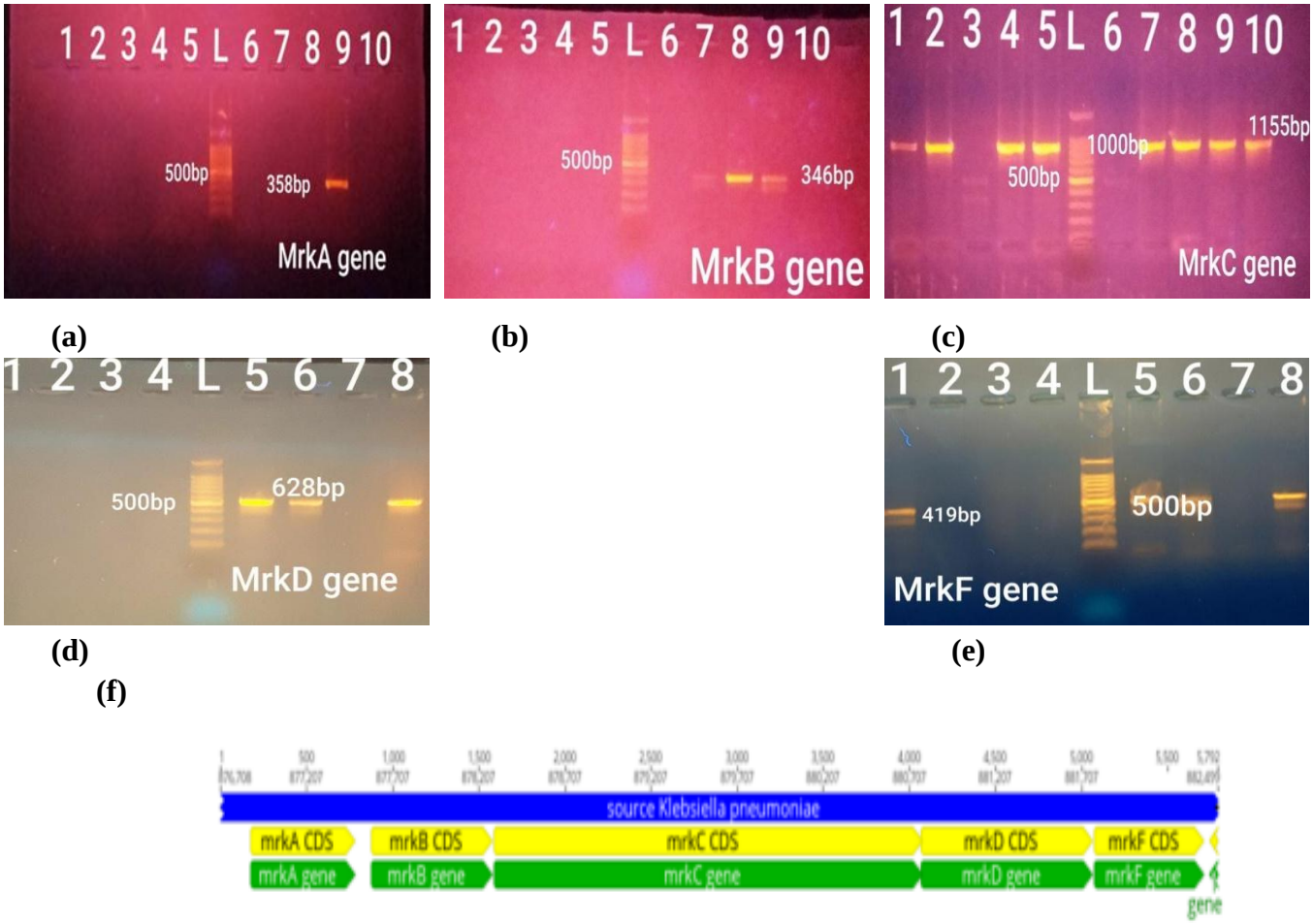

Figure 2. Gel electrophoresis of studied genes. (a) *Mrk A* gene (358 bp). (b) *Mrk B* gene (346 bp). (c) *Mrk C* gene (1155 bp). (d) *Mrk D* gene (628 bp). (e) *Mrk F* gene (419 bp). f: Blast hit resembles the amplified size of *Mrk* gene.

Mrk genes Detection

The results of agarose electrophoresis clarified the precise size of amplified genes for *Mrk* cluster as shown in Table "1". Electrophoresis was applied by using 100 Volt for 45 minutes in 1% agarose. Many isolates of virulent *K. pneumoniae* initiate colonization by using pilus which mediates adhesion environmental and biotic surfaces for facilitating the invading host tissues and promoting the bacterial interaction to form biofilm by the type-3 pilus that are encoded and assembled via *Mrk* genes. In Figure 2(a) to (e), gel electrophoresis bands and blast hit are clarified.

The percentages of *Mrk* genes prevalence were 75, 85, 80, 50, and 60% and 40, 60, 50, 20, and 40% in swab and river samples, respectively as shown in figure "3". In swab samples, *MrkA* gene occurred in 75% of the total twenty isolates. These results are similar to those of Sonbol, *et al*. (2021) and Wyres *et al*. (2020) about occurrence of *MrkA* genes. *MrkB* gene was prevalent in 85% of *K. pneumonia* isolates, while *MrkC* was present in 80% of *K. pneumonia* isolates, and these results agreed with Lue *et al*. (2017) and Al-Rubyaie (2020) who detected the occurrence of *Mrk B* and *Mrk C* genes in hospital samples of *K. pneumoniae*. *Mrk D* genes were present in half of *K. pneumonia* isolates and the results are close to the results obtained by Al-Musawi (2018) and Ragheb *et al.* (2020). *Mrk F* gene was present in 60% of swab isolates. The results matched with Al-Rubyaie (2020) based on presence of *Mrk F* genes.

Prevalence of *Mrk* genes in river water isolates showed higher rates of *K. pneumonia* isolates of *MrkB* (60%), *Mrk C was* (50%), and *MrkA* and *MrkF* were (40%) for both, while *MrkD* gene was the lowest (20%). These results are close to those of Wang, *et al*. (2020), but not similar to the findings of Abd Al-Kareem (2015) and Abdulsada (2020) in which *MrkD* had the highest percentage in river *K. pneumoniae* isolates.

Pilus type-3 are synthesized by expression of *Mrk* genes of *K. pneumoniae* that facilitates the adhesion to cells, including epithelial and endothelial cells besides tissues and injuries with high prevalence and persistence in pathogenic strains especially biofilm former *K. pneumonia* (Al-Oqaili, 2020). The pilus also mediate adhesion to several surface types and abiotic objects like water tanks, pipes, swim pools, air ducts and bathrooms (Caneiras, *et al*. 2019); regarding the dynamic of water or air motion with the other accompanied environmental conditions controlling expression of these pilus or not (Eghbalpoor, *et al*. 2019). The high prevalent pattern for one or more of *Mrk* genes among some *K. pneumonia* isolates may be related to the downregulation and upregulation of specific pilus genes according to the surrounding conditions (Abdulsada, 2020).

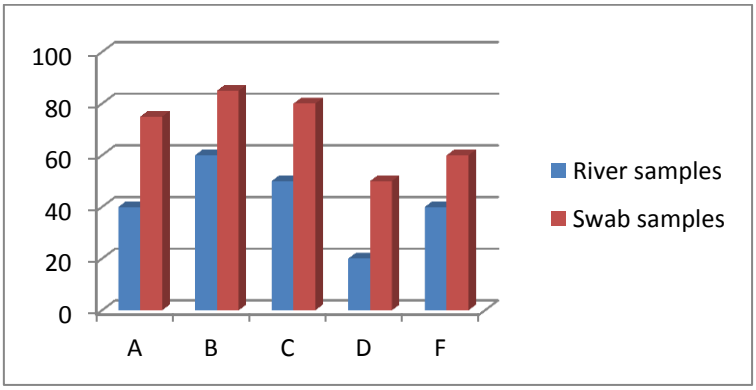

Figure 3. Percentages of *Mrk* genes occurrence with both swab and river strains of *K. pneumoniae*.

Relation of gene prevalence with the sample source

According to the statistical analysis, there was a strong relation of *Mrk* genes presence with the sample type as the LSD was 0.0039 at p-value ≤ 0.01 as the occurrence of certain *Mrk* genes are frequently distributed in swab samples rather than water samples. These results disagreed with Abdal Jabar (2018) who documented the absent significant relationship between gene presence and source of the sample.

The high heterogenous distribution of *Mrk* genes in isolates of *K. pneumoniae* might be as a result of the high genetic diversity and the bacterial differences in both clinical and environmental isolates, which is close to the results of Al-Rubyaie (2020). Table "2" showed the pattern of *Mrk* genes in all tested isolates.

Table2. Gene pattern in both studied groups.

Clinical isolates	***Mrk* genes pattern**	**Environmental isolates**	***Mrk* genes pattern**
1	A-B-F	**1**	B-C
2	A-B-C-F	**2**	A-B
3	A-B-C-D	**3**	B-C-F
4	A-B-C-D	**4**	F-D
5	B-C-D-F	**5**	B-C
6	B-C-F	**6**	B-C
7	A-B-C-D	**7**	B-D
8	A-B-C	**8**	A-F
9	A-B-C-D-F	**9**	A-F
10	B-C	**10**	A-C
11	B-C-D-F		
12	A-D		
13	A-D		
14	A-B-C-F		
15	A-B-C-D		
16	A-B-C-D		
17	A-D-F		
18	A-B-C		
19	A-B-C-D		
20	B-C-D		

The characteristics of clinical *K. pneumoniae* are strongly related and associated to the antibiotic-resistance genes that encoded and expressed by transferable genetic elements or plasmids which are accumulated consequently by *K. pneumoniae* as a result of random antibiotic consumption, differing from plasmids of environmental isolates which are inducible under selective pressure of substances (Wang, *et al*. 2020). On the other hand, the environmental isolates displayed less expressed *Mrk* genes and the pilus biosynthesis are related to inducible plasmids control contributes to the transcriptional/ translational changes and physical interference with water (Silva. *et al*. 2022).

Correlation among *Mrk* genes of all studied samples

Correlations of each couple of genes were achieved by using Pearson coefficient of correlation. *Mrk* genes prevalence was significantly correlated clinical group and but not in environmental samples. As obvious in Tables "3,4" correlations were significant among only *MrkB* and *MrkC* genes in water isolates, whereas there was a strong correlation among *Mrk* genes within the swab samples. The findings obtained in current study are different from those of Muhsin *et al*. (2022) in which not all studied *Mrk* genes had a strong association in their presence.

Table 3. Correlation among *Mrk* genes in clinical isolates of *K. pneumoniae*

	Mrk A	***Mrk B***	***Mrk C***	***Mrk D***	***Mrk F***
MrkA	**-**	**0.005****	**0.011***	**0.018***	**0.018***
MrkB	**0.006****	**-**	**0.016***	**0.023***	**0.023***
MrkC	**0.011***	**0.016***	**-**	**0.050***	**0.050***
MrkD	**0.018***	**0.023***	**0.050***	**-**	**0.0000****
MrkF	**0.018***	**0.023***	**0.050***	**0.0000****	**-**

Table 4. Correlation among *Mrk* genes in environmental isolates of *K. pneumoniae*

	Mrk A	***Mrk B***	***Mrk C***	***Mrk D***	***Mrk F***
MrkA	**-**	**0.217**	**0.597**	**0.135**	**0.808**
MrkB	**0.217**	**-**	**0.028***	**0.288**	**0.395**
MrkC	**0.597**	**0.028***	**-**	**0.355**	**0.783**
MrkD	**0.135**	**0.288**	**0.355**	**-**	**0.673**
MrkF	**0.808**	**0.395**	**0.783**	**0.673**	**-**

The covariant positive relationship which resulted at (p -value less than 0.05 or 0.01) in the genes of the *Mrk* cluster of clinical isolates corresponds to the fact of these genes are arranged in the same transcriptional-orientation which is upregulated in high virulence strains and located within the same operon (*Mrk* operon) induced in response to antibiotic resistance via biofilm formation (Mohammed 2018); while the lack of some associations among *Mrk* genes in *K. pneumonia* isolated from river leads to the overexpression of the *MrkB* and *MrkC* fimbrial genes with a negative inversive relationship with the rest *Mrk* genes (*MrkA, MrkD, and MrkF*) might result by the underlying conditions and entire factors of river water with less persistent of *K. pneumoniae* like biofilm formation and adhesion (Caneiras, *et al*. 2019)

Finally, the non-significant occurrence or negative relationship among environmental genes in comparison to clinical isolates might be strongly downregulated by the heterogeneous expression of some specific bacterial virulence factors and the absence of variety of anti-infectious apparatus like those in human body, which gives the characterized adhesive capability to certain *K. pneumoniae* strains (Al-Timimi, 2021).

CONCLUSION

There was a strong significant relation between the expression of *Mrk* cluster genes and the type of sample either clinical or environmental as the most frequent gene occurrence was only in clinical samples. This fact is depending on prevalence of bacterial virulence profile and persistence of *Klebsiella pneumoniae* of clinical samples; recommending to study the various influencing factors of bacterial virulence in water samples with relation with the environmental related condition in the studied area and water quality parameters.

ACKNOWLEDGEMENT

Great thanks to college of Science in University of Mustansiriyah, to the hospitals staff, and to Environment and Water Directorate- Ministry of science and technology, Baghdad-Iraq, for the support to complete this work.

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