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Detection of *rmpA* and *magA* genes and hypermucoviscosity phenotype in *Klebsiella pneumoniae* isolated from water samples in compare with clinical isolates

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ABSTRACT

From September 2016 to December 2016 thirty eight specimens (urine, blood, burns) were collected in sterilized containers, Sixty five surface water samples were taken from different places. 12/38 (31.57%) of clinical isolates and 20/65 (30.76%) of water isolates were identified as *Klebsiella pneumoniae*. String test was used to determine hypermucoviscosity activity of *K. pneumoni*ae, the results revealed 75 % (15/20) of water isolates and 60% (6/10) of clinical isolates were positive for this test. *magA* and *rmpA* genes that are responsible for hypermucoviscosity of *K. pneumoniae* were detected by using polymerase chain reaction (PCR) technique in clinical and water isolates. *magA* gene was detected in 60% (6/10)of clinical isolates and 15% (3/20) in water isolates, *rmpA* gene was detected in 75% (15/20) of water isolates and 40% (4/10) of clinical isolates, the prevalence of *rmpA* gene in hypermucoviscous clinical isolates were 40% (2/5) and 100% (16/16) in hypermucoviscous water isolates. 18.75% (3/16) of water isolates and 20% (1/5) of clinical isolates which was positive to hypermucoviscosity phenotype found to be having both *magA* and *rmpA* genes, 20% of clinical isolates didn't have neither *rmpA* nor *magA* but found to be positive to hypermucoviscosity phenotype.

Keywords: *Klebsiella*, String test, hypermucoviscosity, *magA*, *rmpA*.

1. INTRODUCTION

Klebsiella pneumoniae has been identified as the most important pathogens causing drug-resistant infections in hospital setting, especially in intensive care unit [1, 2]. Being able to colonize a wide range of body sites, *K. pneumoniae* is traditionally associated with healthcare-related illnesses, including pneumonia, bacteremia, respiratory and urinary tract infections, but additional syndromes (i.e., pyrogenic liver abscess, haemorrhagic colitis, Lemierre's syndromes) occur, which are more often community-acquired, linked to well defined virulence characteristics of select clonal or pathotypes, and require specific screening and/or identification tests [3]. *Klebsiella* are ubiquitous in the environment. They have been found in a variety of environmental situations, such as soil, vegetation, or water, and they influence many biochemical and geochemical processes [4]. They have been recovered from aquatic environments receiving industrial wastewaters, plant products, fresh vegetables, food with a high content of sugars and acids, frozen orange juice concentrate, sugarcane wastes, living trees, and plants and plant byproducts [5].

A high prevalence of *Klebsiella* spp. was measured in rumen, water and soil samples and it was suggested that oral-fecal transmission of bacteria was occurring at the farm [6]. *Klebsiella pneumoniae* isolates have been associated with hyper production of capsular/slime polysaccharide (hypermucoviscosity) and the presence of a putative virulence *magA* gene [7].

The hypermucoviscosity (HMV) phenotype confers resistance to serum complement and to phagocytosis by white blood cells [8]. The two most commonly studied genes associated with the HMV phenotype in *K. pneumoniae* are the regulator of mucoid phenotype *rmpA* and mucoviscosity associated gene *magA* [9]. Another gene associated with hypermucoviscosity phenotype, *rmpA* gene was found to coexist with *aerobactin* gene in HMV-positive *K. pneumoniae* [10].

This study aimed to investigate the frequency of *magA* and *rmpA* genes which are responsible for hypermucoviscosity (HMV) phenotype in *K. pneumoniae* water isolates in comparison with clinical isolates.

2. MATERIALS AND METHODS

2.1 Isolation and Identification of Bacteria

From September 2016 to December 2016 thirty eight specimens (urine, blood, burns) were collected in sterilized containers. Sixty five surface water samples were taken from different places. The collected clinical specimens were streaked directly on MacConkey agar [11], while water samples were isolated by pour plate method [12]. Clinical and water isolates were then cultured on Simmon Citrate agar [13] incubated at 37ºC for 24 hours, then the isolates were subcultured on MacConky agar to obtain pure single isolate. The isolates were identified depending on the morphological features, their response to stain by the Gram stain, and their ability to ferment lactose sugar. The identification was also achieved by using different biochemical tests [13] and by VITEK 2 compact System.

2.2 Hypermucoviscosity test

The hypermucoviscosity phenotype of the *K. pneumoniae* isolates was determined using a modified string test, figure (1), isolates of *K. pneumonia*e were cultured on macConkey agar and incubated for 24 hours at 37 °C. The formation of a viscous string of at least 1 cm was considered positive result [14].

2.3 DNA extraction and estimation of concentration and purity of extracted DNA

DNA extracted from all clinical and water isolates by using Presto™ Mini gDNA Bacteria kit and the concentration and purity of extracted DNA was tested using Nano- drop system.

2.4 Detection of *rmpA* and *magA* genes by Polymerase Chain reaction (PCR)

PCR assay was performed in a monoplex patterns in order to amplify different fragments of genes under study in a single tube for detecting of genes (*magA* and *rmpA,*). The primers listed in table (1) were selected for this study; these primers were provided in a lyophilized form, dissolved in sterile distilled water to give a final concentration of 100 pmol/µL and stored in deep freezer until used in PCR amplification.

Table 1: The primers and their sequences used in conventional PCR for detection of *magA* and *rmpA* genes of *Klebsiella pneumoniae*

Genes	Primer Type	Sequence 5' → 3'	References
magA	*magA*-F	GGTGCTCTTTACATCATTGC	[15]
	magA-R	GCAATGGCCATTTGCGTTAG	
rmpA	*rmpA*-F	ACTGGGCTACCTCTGCTTCA	[9]
	*rmp*A-R	CTTGCATGAGCCATCTTTCA	

2.5 PCR Amplification

The extracted DNA, primers and PCR master mix (promega), were mixed together. PCR mixture was set up in a total volume of 20 µL of master mix kit included 2 µL of each primer, and 3µL of template DNA have been used, the rest volume was completed with sterile de-ionized distilled water, then vortexed. De-ionized water added first, then primers and DNA template added at last .Negative control contained all material except template DNA, so instead that distilled water was added. PCR reaction tubes were centrifuged briefly to mix and placed into thermocycler PCR instrument where DNA was amplified as indicating in the table (2 and 3), these tables showed different programs that used for (*magA* and *rmpA*) genes amplification.

Table 2: Program used to amplify the *magA* gene according to:

Stage	Temperature	Time	Cycle
Initial denaturation	95ºC	5min	35
Denaturation	94 ºC	1 Min	
Annealing	51ºC	1 Min	
Extension	72 ºC	1 min	
Final Extension	72 ºC	10min	

Table 3: Program used to amplify the *rmpA* gene according to:

Stage	Temperature	Time	Cycles
Initial denaturation	95ºC	5min	
Denaturation	94 ºC	1 Min	
Annealing	43ºC	1 Min	35
Extension	72 ºC	1 min	
Final Extension	72 ºC	10min	

3. RESULTS AND DISCUSSION

Bacterial isolates which had the ability to utilize citrate as a source of carbon and energy were tested by VITEK 2 system for accurate identification, for clinical isolates, 12/20 (60%) of isolates were identified as *K. pneumoniae*, 6/20(30%) isolates were identified as *Enterobacter* cloacae, 1/20(5%) isolate was identified as *Cronobacter sakazakii* and 1/20(5%) isolate was identified as *Acinetobacter baumannii*. for water isolates, 20/26(77%) of isolates were identified as *K. pneumoniae*, 4/26(15.4%) isolates were identified as *Enterobacter* cloacae, 1/26(3.8%) was identified as *Aeromonas veronii* and 1/26 (3.8%) was identified as *Klebsiella oxytoca*, (Table 4).

Table 4: Types and percentage of clinical and water isolates tested by VITEK 2

Type of isolates	Type of species	No. of isolates (%)
Clinical isolates	*Klebsiella pneumoniae*	12/20(60%)
	Enterobacter cloacae	6/20(30%)
	Cronobacter sakazakii	1/20(5%)
	Acinetobacter baumannii	1/20(5%)
Water isolates	*Klebsiella pneumoniae*	20/26(77%)
	Enterobacter cloacae	4/26(15.4%)
	Aeromonas veronii	1/26(3.8%)
	Klebsiella oxytoca	1/26 (3.8%)

Hypermucoviscosity is another important surface-related virulence factor of *K.pneumoniae* [7]. *K.pneumoniae* displaying the hypermucoviscosity (HMV) phenotype are considered more virulent than HMV-negative strains [16], so string test was used to determine hypermucoviscosity activity of *K.pneumoni*ae, (Fig.1), 15/20 (75 %) of water isolates were positive and this result didn't agree with Barati and others [17] which recorded (9%) isolates of Aquatic-Borne *K.pneumoniae* demonstrated hypermucoviscosity, 6/10 (60%) of clinical isolates were positive for this test as shown in (Fig.1) and (Table 5), this result agreed with Aljanaby and Alhasani [18] which recorded (62.5%) of clinical isolated *K.pneumoniae* positive for hypermucoviscosity test.

The results showed high percentage of water isolates had hypermucoviscosity phenotype in comparison with clinical isolates, and this indicate that water isolated *K. pneumoniae* was virulence, Almost *K. pneumoniae* produce large amounts of muco-polysaccharide mass and extra-capsular polysaccharides to produce strain with more virulent [19]. In line with this Victor and others [20] 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.

Previous studies have suggested that liver abscesses are caused mostly by HMV-positive *K. pneumoniae*, the HMV phenotype confers resistance to serum complement and to phagocytosis by white blood cells [8].

Figure 1: String test of *Klebsiella pneumoniae*

Table 5: Virulence factors of *Klebsiella pneumoniae* isolates**.**

type of isolates	Hyperviscosity test	
	Positive isolates (%)	Negative isolates (%)
Water isolates	15/20 (75%)	5/20 (25%)
Clinical isolates	6/10 (60%)	4/10 (40%)

3.1 DNA extraction and estimation of concentration and purity of extracted DNA

The concentration of DNA extracted from clinical isolates were (42.61 - 255.9) ng/ml, while DNA extracted from water isolates were (24.5 - 879). The purity of clinical isolates were (1.24- 2.08) ng/ml, while the purity of water isolates were (1.82- 2.09).

3.2 Detection of *rmpA* and *magA* genes and the correlation of this genes with hypermucoviscosity phenotype (HMV) of *Klebsiella pneumoniae*

rmpA (regulator of the mucoid phenotype A) gene is a plasmid-mediated confers a highly mucoviscous phenotype enhanced and regulator of the capsular polysaccharide synthesis [21]. *rmpA*- carrying strains were associated with the hypermucoviscosity phenotype, as well as with the invasive clinical syndrome [22].

Monoplex PCR technique was carried on to detect *rmpA* gene in water and clinical isolates of *K. pneumoniae*. The positive result of *rmpA* gene was confirmed by 1.5% agarose gel electrophoresis stained with red safe stain, electrophoresed in 70 volt for 1 hrs. and photographed under ultraviolet (UV) transilluminator, In this assay, a specific primer was used, the results showed a band of PCR product with 536 bp that represent *rmpA* gene. *rmpA* gene was detected in 15/20 (75%) of water isolates and 4/10 (40%) of clinical isolates, (Fig.2), while *magA* gene was detected in 6/10 (60%) of clinical isolates and 3/20 (15%)of water isolates,(Fig.3) (table 6).

Local study by Al-Jailawi and coworkers [23] recorded *rmpA* gene in 11 isolate (27.5 %) of clinical and environmental isolates, while Barati and others [17] reported that *rmpA* virulence gene was not detected in all of the aquatic-borne *K. pneumoniae* isolates, detection of this gene may indicate the virulence potential of the isolates [24].

The prevalence of *rmpA* gene in hypermucoviscosity clinical and water isolates of *K. pneumoniae* was positive in (85.7%) and negative in (14.3%), this result was corresponding with a study by Si Guo and others [10], Their results showed that hypermucoviscosity (HMV)- phenotype was highly correlated with the presence of the *rmpA* gene, of 14 (HMV)-positive isolates, 85.7 % (12/14) were *rmpA* positive, and 14.3 % (2/14) were *rmpA* negative. None of HMV-negative isolates was *rmpA* positive.

Both *magA* (mucoviscosity-associated gene A) and *rmpA* (regulator of the mucoid phenotype) have been associated with *K. pneumoniae* strains with the HMV phenotype [25], this relationship also reported by other study (26). The *magA* gene has been found to be restricted to K1 isolates, whereas the *rmpA* gene is found in K1, K2, and other serotypes[27].

18.75% of water isolates and 20% of clinical isolates which was positive to hypermucoviscosity phenotype found to have both *magA* and *rmpA* genes, while 40% of clinical positive hypermucoviscosity isolates found to have *magA* gene only, (Table 7). 20% of clinical isolates did not have neither *rmpA* nor *magA* but found to be positive to hypermucoviscosity phenotype, these results corresponding with study by [26] who reported from 177 *K. pneumoniae* isolates, 64 exhibited the HMV phenotype, from positive HMV *K. pneumoniae* isolates, 42 isolates were positive *rmpA* and 15 isolates were positive *magA*. Also reported seven HMV *K. pneumoniae* isolates were negative *rmpA* and *magA*. Other studies examining clinical *K. pneumoniae* isolates have also identified HMV strains that were *rmpA* and *magA* negative. These strains typically possess the K1 or K2 capsular serotype and other genes including *aerobactin*, *kfu*, and *allS* [28]. Thus, it is likely that there are other regulator genes that play a role in the hypermucoviscosity phenotype [26]. .A study by Wiskur *et al*. (2008) reported positive *magA* and *rmpA* genes in positive hypermucoviscous strains while negative HMV strains lacked *magA* and *rmpA*, this study also found in addition to the lack of *rmpA* and *magA* in the HMV negative *K. pneumoniae* strain, two genes (*uge* and *wabG*) were also involved in capsular biosynthesis [19].

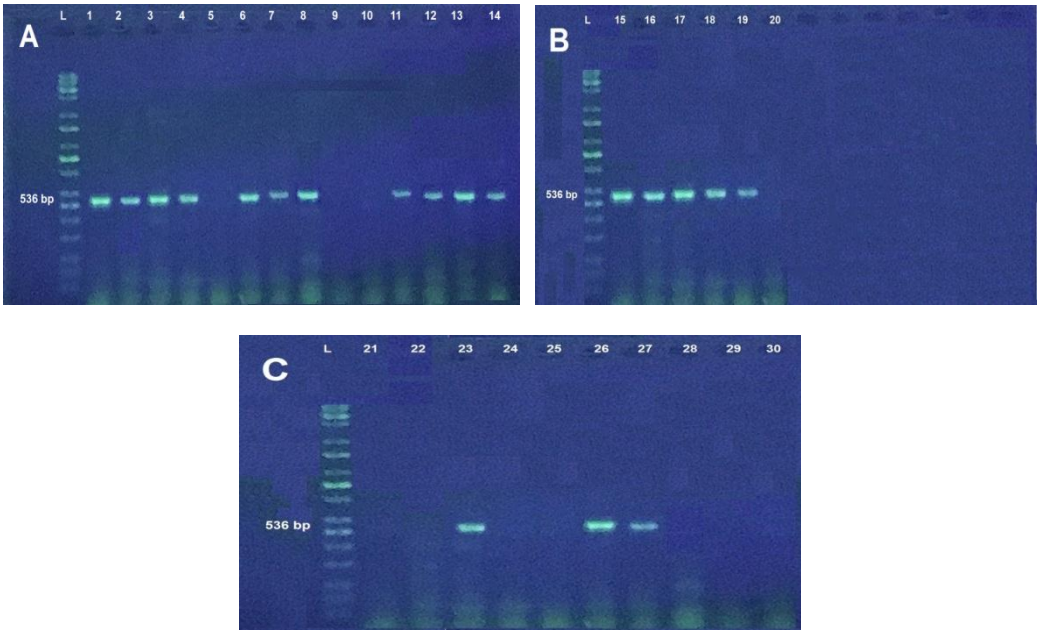

Figure 2: Gel electrophoresis of amplified PCR product of *RmpA* gene (536bp) in monoplex pattern, agarose (1.5%). (A,B) water isolates, (C) clinical isolates, lanes(1,2,3,4,6,7,8,11,12,13,14,15,16,17,18,19) of water isolates were positive to *RmpA* gene ,lanes (23,26,27) of clinical isolates were positive to it,TBE buffer (1x), 70 volt for 1 hrs. Stained with red safe stain. DNA ladder (100 bp).

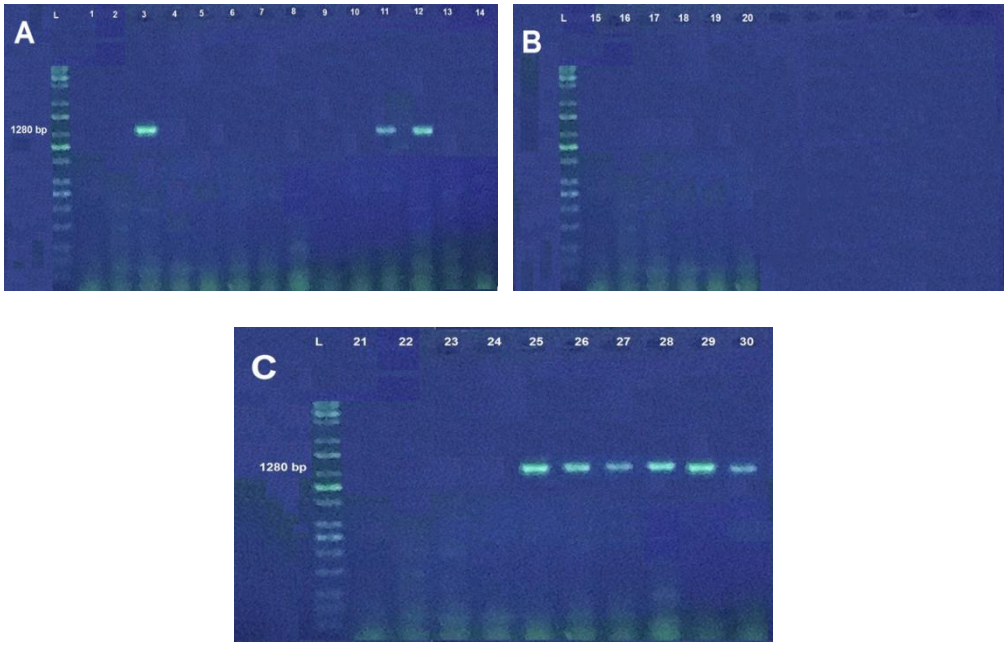

Figure 3: Gel electrophoresis of amplified PCR product of *magA* gene (1280bp) in monoplex pattern, agarose (1.5%). (A,B) water isolates, (C) clinical isolates, lanes (3,11,12) of water isolates were positive to *magA* gene ,lanes (25,26,27,28,29,30) of clinical isolates were positive to it,TBE buffer (1x), 70 volt for 1 hrs. Stained with red safe stain. DNA ladder (100 bp).

Table 6: Distribution of *magA* and *rmpA* genes of *K. pneumoniae* water and clinical isolates.

Virulence genes	Water isolates n (%)	Clinical isolates n (%)	Total
magA	3(15%)	6(60%)	9(30%)
rmpA	15(75%)	4(40%)	19(63.33%)

Table 7: Distribution of *magA* and *rmpA* genes in hypermucoviscous and non hypermucoviscous *K. pneumoniae* water and clinical isolates.

Types of isolates	*magA* n (%)	*rmpA* n (%)	*magA +rmpA* n (%)
Water hypermucoviscous isolates	3 (18.75%)	16 (100%)	3 (18.75%)
Water non hypermucoviscous isolates	0	0	0
Clinical hypermucoviscous isolates	2 (40%)	2 (40%)	1 (20%)
Clinical non hypermucoviscous isolates	4 (80%)	1 (20%)	1 (20%)

4. CONCLUSION

The results revealed that *K. pneumoniae* water isolates possess virulence factors commonly present in the clinical isolates. Most clinical and water isolates were hypermucoviscous isolates, Hypermucoviscosity genes (*magA* and *rmpA*) were found in both clinical and water isolates .The results suggested that *K. pneumoniae* water isolates could be potentially virulent to humans based on the phenotypic and genotypic characterization.

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