ORIGINAL ARTICLE

Detection of Biofilm and Siderophore Encoding Genes Implicated in the Pathogenesis of *Klebsiella pneumoniae* Isolated from Different Clinical Specimens

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ABSTRACT

Background: *Biofilm and siderophores protect K. pneumoniae, making them resistant to antimicrobials and increase the ability to evade immune system.* ***Objective:*** *The aim of the present study was to detect some of the virulence genes responsible for biofilm formation and siderophore production implicated in the pathogenesis of K. pneumoniae.* ***Methodology:*** *Crystal violet method was used to detect biofilm phenotypically, hypermucoviscosity by string test and virulence genes were detected by PCR.* ***Results:*** *Phenotypically, biofilm formation was positive in 76% and negative in 24%. Genotypically, FimH and ycfm were detected in 100% of isolates while mrkD in 98%. Kfu, entB and iutA were found in 100%, 68% and 34% respectively. Phenotypically by string test 66% of isolates were classified as hypermucoviscous, while 34% as classical. rmpA gene was detected in 52% of isolates.* ***Conclusion:*** *FimH, mrkD, ycfm, Kfu and iutA genes are implicated in multisystemic infection of K. pneumoniae. Enterobactin gene is more predominant than other siderophore genes.*

INTRODUCTION

Klebsiella pneumoniae is a leading cause of nosocomial and community acquired infections of human, including urinary tract infections, surgical sites infections, soft tissues infections, bacteremia and pneumonia[1].

There are two major types of *K. pneumoniae*; hypervirulent (hvKp) and classical (cKp), the latter is the most common and less severe subtype. The hvKp strains possess hypermucoviscosity and are responsible for many infections in immunocompetent and in diseased patients [2].

Moreover, cKp is rapidly acquiring resistance to all known antibiotics, thus becoming increasingly difficult to treat. The production of extended spectrum β-lactamases and carbapenemases are two main resistance mechanisms among *K. pneumoniae*. Nevertheless, multidrug resistance (MDR) (resistant to three or more antimicrobial classes) and extensive drug resistance (XDR) resistance to at least one agent in all but two or less antimicrobial classes) are common rendering severe infections with cKp strains like pneumonia and bloodstream infections life-threatening [3].

Many factors participate in virulence and pathogenicity of *K. pneumoniae* which allow it to overcome innate host immune response and to cause infection in the human host, such as the capsule, lipopolysaccharide, iron-scavenging systems, pili and adhesions [4].

Iron is critical for the function of many cellular processes [5]. Therefore, iron acquisition systems like iron chelator siderophore are detrimental for the growth of pathogenic bacteria[6]. It allows bacteria to take up protein-bound iron from the host cells[7]. Siderophores are small, high-affinity iron chelators produced by many microorganisms. Several siderophores have been previously detected in klebsiella like Enterobactin (Ent), Salmochelin (Sal), Yersiniabactin (Ybt) and Aeribactin (Aer) [8].

The formation of biofilms by *K. pneumoniae* is a key player in facilitating evasion of host defense mechanisms, communication between bacterial cells and protection against antibiotic action. The bacteria's capability to produce biofilms depends on multiple genetic factors. Therefore, surface components of the bacterial cell that increase the efficiency of biofilm formation are likely to play a major role in the establishment of infection by pathogens [9].

Researchers postulated that virulence gene analysis is very important to help in understanding *K. pneumoniae* infections [10,11]. Therefore, this study aimed to detect some of the virulence genes that are responsible for biofilm formation and siderophore production which are implicated in the pathogenesis of *K. pneumoniae* isolated from different clinical specimens.

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METHODOLOGY

All culture media were purchased form Oxoid (UK) and antimicrobial discs were purchased from BioRad (UK)

Bacterial strains

Fifty *K. pneumonaie subsp. pneumonaie* were obtained from different clinical specimens including blood, urine, sputum, minibal and wound swab. Isolates were all Gram-negative bacilli, catalase positive, oxidase negative, ferment glucose and lactose, urease positive, Indole negative, Voges Proskauer positive, methyl red negative, citrate positive and immotile. Bacterial isolates were stored in Luria Bertani (LB) broth containing 30% glycerol and stored at -80°C. For bacterial revival, one loopful was streaked over blood agar and incubated at 37°C [12].

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was carried out by Kirby-Bauer method[13]. Antibiotics discs were chosen according to the Clinical and Laboratory Standards Institute (CLSI) [14].

Phenotypic detection for ESBL

ESBL production was determined according to the CLSI guidelines using a disc of cefotaxime (CTX) alone and cefotaxime-clavulanate (CCT) and a disc of ceftazidime (CAZ) alone and ceftazidime-clavulanate (CZA). An increase of zone diameter ≥5 mm for either antimicrobial agent in combination with clavulanate versus the zone diameter of the antimicrobial when tested alone is categorized positive for ESBL production [15].

Phenotypic detection of biofilm formation

Biofilm formation was detected as described before[16]: Briefly, an overnight (16 – 20 hours) culture of test isolate in LB broth was diluted 1:100 in fresh LB medium and 100 µl were inoculated in triplicate in 96-well flat bottom polystyrene plate. Wells inoculated with LB medium without bacteria were used as negative control. After 24 hours of static incubation at 37°C, the culture suspensions were removed. Each well was gently rinsed twice using 200 µL of sterile saline solution. The formed bacterial biofilm was fixed using 200 µl methanol for 30 minutes. The fixed biofilm was stained with 100 µL of 2% crystal violet for 15 min. The plate was washed using distilled water and dried at room temperature for 10-15 minutes. Solubilization was done in 200 µL of 33% acetic acid for 10 minutes. The amount of crystal violet bound to biofilm was evaluated by measuring the optical density (OD) at 590 nm by ELISA reader (TECAN, Switzerland). The cut-off OD (ODc) was calculated as three standard deviations above the mean OD of the negative control wells. Isolates with mean OD less than ODc were considered non adherent; and those with mean OD greater than ODc, were considered biofilm positive.

Phenotypic detection of hypermucoviscosity

Hypermucoviscosity of *K. pneumoniae* isolates was identified using modified string test [17]. The formation of a mucoviscous string of >5mm on a bacteriology inoculation loop used to stretch a colony grown overnight on blood agar plate at 37◦C was considered string test positive. If the stretch of colony ≥5 mm the strain was considered hvKp and if the stretch of colony < 5 mm the isolate was considered cKp.

Genotypic detection of *K. pneumoniae* virulence factors

Bacterial genomic DNA was extracted by boiling technique [18]. Briefly, 2 to 3 colonies from an overnight culture were suspended in TE buffer containing 0.1% triton X100. *K. pneumoniae* suspensions were incubated in a boiling water bath for 10 minutes followed by rapid cooling on ice. After centrifugation for 15 minutes at 14,000 rpm in a microfuge, the supernatant was separated and processed as the source for bacterial DNA.

In this work, genes used for the identification of biofilm formation were *fimH* (Type 1 fimbrin D-mannose specific adhesin), *mrkD* (The type 3 fimbrial adhesin) and *ycfm* (Penicillin-binding protein activator, *lpoB*). Genes for the detection of siderophores were *entB* (enterobactin synthase component B), *kfu* (Iron uptake system gene) and *iutA* (Ferric aerobactin receptor). Finally, *rmpA* gene (regulator of mucoid phenotype A) was utilized for molecular detection of hvKp.

All PCR reactions were excuted in 25 µl final volume containing 12.5 µl hot start PCR master mix MyTaqTM HS Red Mix (BioLine Scientific, London, UK), 10 pmole of each primer (all primers were purchased from Thermo Fisher Scientific, California., USA) (Table I) and 1 µl bacterial DNA. A negative control was prepared by the addition of the exact contents to the tube with water instead of the extract. All PCR reactions were executed on Veriti thermal cycler (Applied Biosystems, California, USA). Three Multiplex PCR reactions were performed, where 2 genes were detected together (*fimH, mrkD*) (*ycfm, entB*), (*kfu, iutA*), while *rmpA* gene in a single plex reaction. PCR products were separated by gel electrophoresis on 1.7% agarose gel containing 0.5 µg/ml ethidium bromide.

Table 1: Primer used in PCR reactions

Primers	Sequence 5' – 3'	Annealing °C	Amplicon (bp)	
fimH-F	TGCTGCTGGGCTGGTCGATG	58	688	[19]
fimH-R	GGGAGGGTGACGGTGACATC			
mrkD-F	TTCTGCACAGCGGTCCC	58	240	[19]
mrkD-R	GATACCCGGCGTTTTCGTTAC			
ycfm-F	ATCAGCAGTCGGGTCAGC	58	160	[19]
ycfm-R	CTTCTCCAGCATTCAGCG			
entB -F	ATTTCCTCAACTTCTGGGGC	58	371	[19]
entB-R	AGCATCGGTGGCGGTGGTCA			
kfu-F	GAAGTGACGCTGTTTCTGGC	58	797	[20]
kfu-R	TTTCGTGTGGCCAGTGACTC			
iutA-F	GGCTGGACATCATGGGAACTGG	58	300	[19]
iutA-R	CGTCGGGAACGGGTAGAATCG			
rmpA-F	ACTGGGCTACCTCTGCTTCA	50	535	[21]
rmpA-R	CTTGCATGAGCCATCTTTCA			

RESULTS

K. pneumoniae strains were isolated from 25 (50%) males and 25 (50%) females. Seventeen (34%) were collected from blood and urine samples each, 7 (14%) from sputum, 6 (12%) from wound swabs and only 3 (6%) from minibal samples.

Antimicrobial susceptibility testing

Antimicrobial resistance varied among the 50 *K. pneumoniae* isolates (Table 2). Resistance to cell wall synthesis inhibitors ranged from 28% to Meropenem to 92% to Amoxicillin/ clavulanic acid. Resistance to Piperacillin / tazobactam resistance was 50% while resistance to third generation cephalosporin ranged from 78%-82%. Resistance to cefoxitin was detected in 44% of isolates. ESBL production was confirmed in 30 (60%) of isolates using combined disc diffusion method.

As regards protein synthesis inhibitors, highest resistance was detected with Minocycline (60%) and the lowest with Amikacin (36%). Resistance to fluoroquinolone was 38% against Levofloxacin and 46% against ciprofloxacin. Resistance to Trimethoprim/ sulfamethoxazol was 64 %. Nitrofurantoin was sensitive in 50% only. Multidrug resistance (MDR) was confirmed in in 60% of isolates.

Table 2: Antimicrobial Susceptibility testing of the 50 *K. pneumoniae* isolates by disc diffusion metho

Antimicrobial agents	Sensitive		Intermediate		Resistant	
	No	%	No	%	No	%
Ampicillin/ sulbactam	5	10.0	0	0.0	45	90.0
Amoxicillin/ clavulanic acid	4	8.0	0	0.0	46	92.0
Piperacillin / tazobactam	23	46.0	2	4.0	25	50.0
Cefoxitin	25	50.0	3	6.0	22	44.0
Cefuroxime	11	22.0	0	0.0	39	78.0
Ceftazidime	14	28.0	0	0.0	36	72.0
Cefotaxime	11	22.0	0	0.0	39	78.0
Ceftriaxone	10	20.0	0	0.0	40	80.0
Cefepime	9	18.0	0	0.0	41	82.0
Meropenem	33	66.0	3	6.0	14	28.0
Ertapenem	31	62.0	0	0.0	19	38.0
Imipenem	29	58.0	0	0.0	21	42.0
Amikacin	28	56.0	4	8.0	18	36.0
Tobramycin	29	58.0	2	4.0	19	38.0
Minocycline	13	26.0	7	14.0	30	60.0
Levofloxacin	28	56.0	3	6.0	19	38.0
Ciprofloxacin	26	52.0	1	2.0	23	46.0
Trimethoprim/ Sulfamethoxazol	18	36.0	0	0.0	32	64.0
Nitrofurantoin	23	46.0	2	4.0	25	50.0

Detection of Biofilm formation

Phenotypically, biofilm was detected in 38 (76%) and negative in 12 (24%). Genotypically, *fimH* and *ycfm* genes were detected in 100% of isolates while *mrkD* in 98% of isolates (Figure 1).

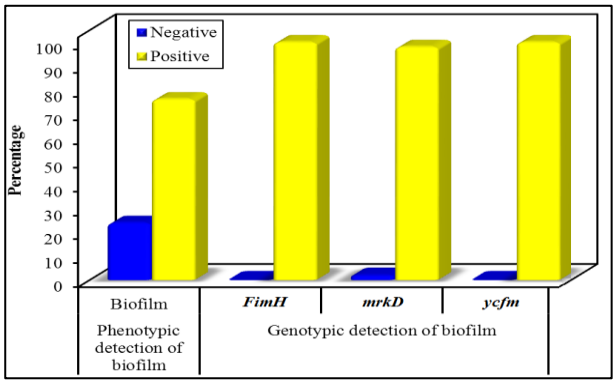

Fig. 1: Phenotypic and genotypic detection of biofilm formation by *K. pneumoniae* isolates

Detection of Hypermucoviscosity

Phenotypically by string test 33 (66%) of isolates were categorized as hypermucoviscous, while 17 (34%) as classical isolates. Genotypically, *rmpA* gene was identified in 26 (52%) of isolates. In comparing hypermucoviscous parameters; 23 (46%) were positive for both string and *rmpA* gene and 11 (22%) were negative for both. However, 3 (6%) were string test negative but positive for *rmpA* gene and only 13 (26%) were negative for *rmpA* gene and positive for string test.

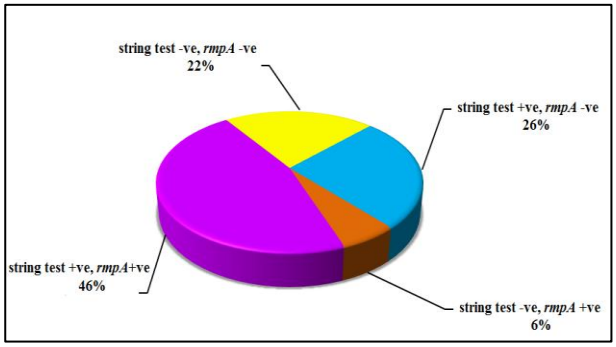

Fig. 2: Association between hypermucoviscous parameters: rmpA gene and string test

Detection of Siderophores formation:

Siderophore genes *kfu*, *entB* and *iutA* were identified in 100%, 68% and 34% respectively among 50 isolates using PCR.

Association between *rmpA* and *iutA* genes:

Out of 50 *K. pneumoniae* isolates 13 (26%) were positive for both *rmpA* and *iutA* genes and 13 (26%) were negative. On the other hand, 4 (8%) were *iutA* negative but positive for *rmpA* gene and 20 (40%) were negative for *rmpA* gene and positive for *iutA* gene (Table 3).

Table 3: Relation between rmpA gene and iutA gene

	rmpA-positive		rmpA-negative		Total	
	No	**%**	**No**	**%**	**No**	**%**
iutA-Positive	13	26	20	40	33	66
iutA-Negative	4	8	13	26	17	34
Total	17	34	33	66	50	100

There was no significant analysis correlation between *rmpA* gene with different categories of resistance (p= 0.643) Table (IV) or between *iutA* and *Kfu* genes with different categories of resistance (p= 0. 273) (p= 0. 059) respectively (Table 4).

Table 4: Association between virulence genes and antimicrobial resistance profile

	MDR (n = 30)		Non-MDR (n = 20)		χ^2	P
	No.	**%**	**No.**	**%**		
rmpA	17	56.7	10	50.0	0.215	0.643
iutA	18	60.0	15	75.0	1.203	0.273
Kfu	24	80.0	11	55.0	3.571	0.059

χ^2: Chi square test

p: p value for comparing between the studied categories

Virulence genes distribution among clinical specimen:

fimH, *mrkD*, *ycfm* and *entB* were predominant in all sample's types (100%), while *iutA* gene was common in sputum and wound swab 100 and 83.3% respectively. *kfu* gene was prevalent in urine (76.5%), minibal (66.7%) and wound (66.7%). However, *rmpA* gene was most abundant in minibal (66.7%) and blood sample (58.8%) (Table 5).

Table 5: Distribution of virulence genes coding for biofilm among different clinical specimen

	Minibal (n = 3)		Blood (n = 17)		Sputum (n = 7)		Urine (n = 17)		Wound (n = 6)	
	No.	%	No.	%	No.	%	No.	%	No.	%
FimH	3	100	17	100	7	100	17	100	6	100
mrkD	3	100	17	100	7	100	17	100	6	100
Ycfm	3	100	17	100	7	100	17	100	6	100
entB	3	100	17	100	7	100	17	100	6	100
iutA	1	33.3	11	64.7	7	100	9	52.9	5	83.3
Kfu	2	66.7	11	64.7	4	57.1	13	76.5	4	66.7
rmpA	2	66.7	10	58.8	4	57.1	8	47.1	2	33.3

DISCUSSION

The extensive use of antimicrobials led to high level of resistance in *K. pneumoniae* [22]. In this study, the resistance categories were distributed as MDR in 60% of isolates and non-MDR in 40%. Higher resistance levels were observed by Wasfi et al.[10], and Aljanaby et al.[23], who revealed that MDR was noted in 77.7% and 84.4 respectively. The high rates of antimicrobial resistance reported in this work can be explained by the lack of strict policies that govern the use of antimicrobials in Egypt[24,25].

Biofilm protects bacteria from both antibiotic effect and host immune responses[26]. Phenotypic detection of biofilm in this study was positive in 76% of isolates while negative in 24%. This finding was in accordance with El Fertas et al [27] who identified biofilm formation phenotypically in 88.8%. Genotypic detection involved the use of *fimH*, *mrkD* and *ycfm* genes, where *fimH* and *ycfm* were detected in 100% of isolates and *mrkD* was detected in 98%. This highly correlates with Aljanaby et al[23] who demonstrated that both *fimH* and *ycfm* were found in all isolates (100%). On the contrary, Kuş et al[28] reported lower detection of *fimH*, *mrkD*, *ycfm* genes in 64.2%, 83% and 86.8% of isolates respectively. The discrepancies in detection levels of these genes may be associated with the difference in the geographic areas.

fimH plays an important role in *K. pneumoniae* infections[29]. *mrkD* is a type 3 fimbriae which allow adhesion to different human tissues; lung, bladder, kidney and is a robust promoter of biofilm formation on abiotic surfaces and contribute in biofilm associated infections[30]. *ycfm* is an extracellular matrix binding outer lipoprotein membrane [31]. In the current study, the genotypic detection of biofilm genes was more sensitive and subtle than phenotypic detection, where only 76% isolates were positive phenotypically while only 2% of the 50 isolates was negative genotypically.

There are three main siderophore systems among Enterobacteriaceae: aerobactin, enterobactin, and yersiniabactin [32]. In this study aerobaction was detected by *iutA* gene. Enterobactin by *entB* and iron transport system by *kfu* gene.

Aerobactin has a low affinity for free ferric iron (Fe3+) [33]. In this study *iutA* gene was only detected in 34% of isolates. Enterobactin is a siderophore compound produced by bacterial cells for iron uptake from iron-binding proteins of the host, and it has strong affinity to extracellular ferric chelators [34]. *entB* was identified in all (100%) isolates. Similar results were highlighted by several studies like Kuş et al[28], Aljanaby et al [23]who demonstrated that *entB* gene was detected in 96.2% and 100% respectively.

Kfu gene is a regulator of iron transport system; associated with capsule formation, hypermucoviscosity, purulent tissue infection[35]. In the present study, *kfu* was detected in 68% of isolates. This was similar to Aljanaby et al [23] who detected *kfu* gene in 65.6%, while Shakib et al [36] detected it in only 11.4%.

EntB, and *iutA* genes are widely disseminated among *K. pneumoniae* strains [11]. However, *entB* is only associated with virulence when it occurs in association with *iutA* or *kfu* [37]. In the current study, all *K. pneumoniae* carried the *entB* gene; however, the existence of the genes encoding *entB* in combination with *iutA* and *kfu* was found in only 66% and 68% respectively.

Hypervirulent *K. pneumoniae* produce more aggressive disseminated infections than cKp strains. In present study 66% of isolates were classified as hvKp, while 34% as cKp phenotypically. This finding is in agreement with Tan et al.[21] and Aljanaby et al [23], who stated that 42.6%, 62.5% of their strains respectively were hypermucoviscous. In disagreement with this study lower percentages were reported by Shakib et al [36] and El Fertas et al.[27] who noticed hypervirulent strains in 14.3% and 9.2% respectively.

rmpA gene can positively control the mucoid phenotype of *K. pneumoniae* that is found only in hvKp strains and located on a plasmid [38]. In the present study *rmpA* gene was detected in 52% of isolates. This correlated with Aljanaby et al [23] and Candan et al [19],who reported that 62.5%, 66.6% of their strains were hypermucoviscous isolates respectively. However, Shakib et al.[36], confirmed that only 5.7% were positive for *rmpA* gene.

In this study, 46% of *K. pneumoniae* isolates were positive for both string test and *rmpA* gene and 22% were negative. On the other hand, 6% were string test negative but positive for *rmpA* gene and only 26% negative for *rmpA* gene were string test positive. However, Tan et al.[21], illustrated that *rmpA* was dramatically associated with a positive string test where *rmpA* was detected in 45.7% and string test was positive in 42.6% of all *K. pneumoniae* isolates. This can be explained by the presence of other genes that are involved in hypermucoviscosity for *K. pneumoniae* including transcriptional regulators in serotype-specific extracapsular polysaccharide production (*rmpA2*) and mucoviscosity-associated gene (*magA*) [39].

iutA and *rmpA* gene both are located on plasmid (Large Virulence Plasmid of *K. pneumoniae* pLVPK) [40]. In this work, both *rmpA* and *iutA* genes were positive in 26% of isolates and both negative in 26% as well. Guo et al.[41] identified *iutA* gene and *rmpA* in 86.9% and 92.9% of hypermucoviscous isolates respectively.

fimH and *ycfm* biofilm formation genes were identified in all types of samples. This finding highly correlated with Aljanaby et al.[23], who illustrated that the two genes were found in all types of samples including urine, blood and tissue (100%).

mrkD was detected in 100% of minibal, blood, sputum and wound samples and in 94.1% of urine samples. This finding correlated with Wasfi et al[10], finding who stated that *mrkD* was prevalent in all types of samples including blood, urine and sputum.

In this study, *entB* gene was detected in all type of samples and this highly correlated with Aljanaby et al[23], who reported that *entB* was prevalent in all types of samples (100%). This result showed that this iron chelator gene is identified in almost all *K. pneumoniae* clinical isolates with all types of infection.

Kfu gene was found in 76.5% of urine samples, in 66.7% of wound swab and minibal samples, in 64.7% in blood samples and 57.1% in sputum samples. *K. pneumoniae* that infect urinary tract use *kfu* for chelating iron more than when infection is present elsewhere in the body [35].

rmpA, hypermucoviscosity gene, was abundant in minibal and blood sample 66.7 and 58.8% respectively and less in sputum, urine and wound swab 57.1%, 47.1 % and 33.3% respectively. Kuş et al [28] did not detect *rmpA* gene in any of urine, blood, wound, drainage fluid, broncho-alveolar lavage and cerebrospinal fluid samples. *rmpA* contributes to preventing the phagocytosis and opsonophagocytosis of *K. pneumoniae* by the immune cells and inhibits complement-mediated lysis and opsonization. Thus, *rmpA* regulation is associated with the escape of *K. pneumoniae* from immune responses [35].

CONCLUSION

K. pneumoniae is highly resistant to a wide spectrum of antimicrobials such as fourth generation cephalosporins, so possibly in near future there will not be any proper antibiotics for this bacterium. Abuse and misuse of broad-spectrum antibiotics is the main reason. On the contrary, Carbapenems, especially Meropenem are still active in more than 72% against *K. pneumoniae*[42]. As regards biofilm formation; genotypic detection was more sensitive than phenotypic detection. *fimH*, *mrkD*, and *ycfm* genes are implicated in multisystemic infection of *K. pneumoniae*. Enterobactin gene (entB) is more predominant than other siderophore genes (*iutA*, *kfu*). Moreover, *kfu* and *iutA* genes are involved in systemic infection of *K. pneumoniae*.

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- Each author listed in the manuscript had seen and approved the submission of this version of the manuscript and takes full responsibility for it.
- This article had not been published anywhere and is not currently under consideration by another journal or a publisher.

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