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Investigation of the genic variation in *MrkA* gene in forming and non-forming biofilm isolates of *Klebsiella pneumonia*

Shaymaa Fouad Rasheed Al-Khazraji, Mohammad Abdul Rahmman Mohammad Al-Maeni* and Suaad Ali Ahmed

Department of biology, College of science, University of Baghdad, IRAQ

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Abstract

Biofilm formation is a powerful strategy harbored by *Klebsiella pneumonia* isolates and helps them prevent antimicrobials from penetrating the cell and escaping the immune system. The aim of the study to correlate the genic variation of *MrkA* gene in isolates of constructing and non-constructing biofilm. The result showed that 55% of isolates formed biofilm, and their Minimum Bactericidal Concentrations (MBC) were significantly higher than the MBC of non-forming biofilm isolates. In addition, the MBC mediating biofilm destruction was higher than the Minimum Inhibitory Concentration (MIC) mediating planktonic cell killing. This may indicate that the biofilm plays a significant role in the survival of *K. pneumonia* isolates in their host. Moreover, *MrkA* gene was only determined in several biofilm-forming isolates, which may indicate their role in forming biofilm. However, studying the genic variation revealed that *MrkA* gene was conserved, and no variation was detected at all through comparing DNA sequencing of *MrkA gene* between isolates and the reference genome. Finally, we think *MrkA* genes participate in biofilm formation, which helps isolates persist for a long time in their host.

Keyword: Klebsiella pneumonia, *MrkA* gene, biofilm formation

دراسة التغاير الجيني في جين *MrkA* في العزلات المكونة وغير المكونة الى الاغشية الحيوية لبكتيريا كليبسيلا الرئوية

شيماء فؤاد رشيد, محمد عبد الرحمن محمد* , سعاد علي احمد

قسم علوم الحياة, كلية العلوم, جامعة بغداد, العراق

الخلاصة

يعد تكوين الأغشية الحيوية استراتيجية قوية تؤويها عزلات الالتهاب الرئوي ال الكَلِبْسية الرئوية وساعدتها في منع مضادات الميكروبات من اختراق الخلية والهروب من الجهاز المناعي. كان الهدف من الدراسة هو الربط بين التباين الجيني لجين *MrkA* في العزلات المكونة وغير المكونة الأغشية الحيوية. أظهرت النتائج أن 55% من العزلات شكلت الأغشية الحيوية وكان MBC لديها أعلى بكثير مقارنة مع العزلات غير المكونة للأغشية الحيوية. بالإضافة إلى ذلك، كان تدمير الأغشية الحيوية بوساطة MBC أعلى من تثبيط الخلايا العوالق بوساطة MIC. قد يشير هذا إلى أن الغشاء الحيوي يلعب دورًا حاسمًا في بقاء عزلات الالتهاب الرئوي الكَلِبْسية الرئوية في مضيفها. علاوة على ذلك، تم تحديد جين *MrkA* فقط في عدة عزلات تشكل الأغشية الحيوية وهذا قد يشير إلى دورها في تكوين الأغشية الحيوية. ومع ذلك، أظهرت دراسة التباين الجيني أن الجين

* Email: mhogene79@yahoo.com

*MrkA*غير متغاير ولم يتم اكتشاف أي تباين على الإطلاق من خلال مقارنة تسلسل الحمض النووي لجين *MrkA*بين العزلات والعزلة مع الجينوم المرجعي. أخيرًا، نعتقد أن جين *MrkA* تشارك في تكوين الأغشية الحيوية التي تساعد العزلات على البقاء لفترة طويلة في مضيفها.

Introduction

Klebsiella pneumonia is one of the bacteria that behave as opportunistic pathogens in the host , and their presence is correlated with nosocomial infections by which it causes pyogenic liver abscess, urinary tract infections, and pneumonia. *K. pneumonia* becomes resistant to many antimicrobial agents with different classes, which are cephalosporins, aminoglycosides, and fluoroquinolones [1]. *K. pneumonia* possesses the carbapenemase genes that show tolerance to β-lactam antibiotics, and these genes can be easily transferred from environmental bacterial strains to pathogenic strains [2, 3]. Many genes mediating antibiotic resistance, such as ESBL and carbapenemases genes, are harbored on the plasmid; therefore, they are readily transferred among the bacterial strains community [4, 5]. One of the defense strategies of this bacterium is its capacity to construct a biofilm that is considered a barrier preventing antimicrobial agents from penetrating the community of bacterial cells; therefore, it is considered a multidrug resistance pathogens causing disease with a high mortality rate [6, 7].In general, investigation of the variation in the whole genome of isolates of different species is important to detect the type of scheme responsible for the persistence of these isolates in their host [8] . *K. pneumonia* harbors different virulence factors that aid this bacterium in invading the host and escaping the immune system. These factors are polysaccharide capsule, pili, and lipopolysaccharides. However, biofilm is the major virulence factor enrolled in antibiotic and immune system resistance [9-11]. Genetic factors participate in the initiation and maturation of biofilm in *K. pneumonia* and are classified as type I fimbriae with *fimH* gene, polysaccharides and adhesins with *pgaC* gene, capsular polysaccharide with *wza* gene [12-15]. Adherence of *K. pneumonia* in tissue host is achieved by protein structures known as type 1 and type 3 fimbriae that participate in pathogenicity [16, 17]. *MrkA* gene encode to MrkA protein in *K. pneumonia* that form type 3 fimbriae and facilitate binding of the bacteria to host mucosal surfaces and formation of biofilm [18-20]. *MrkA* gene encode to Type 3 fimbriae while *mrkD* encodes the adhesin of Type 3 fimbriae [21, 22]. Most of studies achieved on *MrkA* gene was correlated with the prevalence of this gene among different isolates forming biofilm and resisting to bactericidal agents. However, detection the variation of *MrkA* gene among different strains rarely investigated by the researchers especially between isolates constructing and non-constructing biofilm. Therefore, the study aimed to investigate the variation in *MrkA* gene in biofilm forming and non-forming isolates. In addition, green tea contains Polyphenol EGCG, which is a powerful agent that inhibits biofilm formation through inhibition of the construction of fiber assembly by interacting with genes responsible for the formation of a matrix of polymer of biofilm [23]. Therefore, we intended to visualize the effect of green tea on biofilm formation in the current study.

Materials and methods

Isolates under the study

The 20 collected isolates of *K. pneumonia* from different hospitals in Baghdad city were diagnosed by plating the isolates in MacConkey agar and blood agar. In addition, indole production, urease test, hydrogen sulfide production, and motility were performed as chemical tests to differentiate between *K. pneumonia* and other bacterial isolates. Moreover, *16sRNA* sequencing was conducted to ensure the identity of the *K. pneumonia* isolates. The ethics committees authorized this work under the reference number CSEC/1023/0093.

Protocol of biofilm construction assay

Inoculation of 20 *K. pneumonia* isolates in tryptic soy broth contains glucose (1% concentration) and left at 37 °C for 24 h and this time is sufficient for bacteria to engage with stages of biofilm formation. Then 200 µl of the mixture (including 180 µl of media and 20 µl overnight-bacterial culture) was loaded on a microtitre plate handling 96 wells and hold at a temperature of 37 °C for 24 h. 0.2 ml of distilled water (DW) was used to wash the wells and kept in an inverted position on filter paper for the purpose of drying. Subsequently, 180 µl of crystal violet (0.1%) was added to the wells for staining, and then the destaining process was performed with the addition of acetic acid. ELISA was used to measure the OD of each isolate in the wells, and the reading was taken in triplicate at 570 nm wavelength for each well with a particular isolate, while the reading of negative control was represented by taking the OD of the medium without the isolate and reading of positive control represented by taking OD of isolates without media. Three standard deviations plus the mean OD of the negative control is referred to as ODc while subtracting the average OD of the triplicate of each isolate from the OD of control, which is referred to as ODi. Non-biofilm-construction, weak-biofilm-construction, moderate-biofilm-construction, and strong-biofilm-construction were detected when ODi < ODc, ODc < ODi < or = 2*ODc, 2*ODc < ODi < or = 4*ODc, and 4*ODc < ODi respectively [24, 25].

Achievement of MBC on biofilm

After the construction of the biofilm, the planktonic cells were discarded from each well. Then, each well of biofilm with a particular isolate was treated with serial dilution of antimicrobial agents (ciprofloxacin :2.5-160 mg/ml, gentamicin :12.5-800 mg/ml, and Tobramycin:6.25-400 mg/ml) and kept for one day. Later, MBC was detected by culturing treated biofilm with antibiotics in each well on nutrient agar plates. The plate with no growth indicates MBC for the biofilm in each isolate.

Effect of antibiotics on biofilm construction

The isolates that were detected previously as biofilm producers and non-biofilm producers were incubated with TSB containing glucose with 1% concentration to form bacterial broth. 100 µl of bacterial broth was loaded to a microtitre plate containing 96 wells. Antibiotics with 100 µl and serial dilution (ciprofloxacin :2.5-160 mg/ml, gentamicin :12.5-800 mg/ml, and tobramycin :6.25-400 mg/ml) were added to the 96 wells and mixed with bacterial growth. The wells within the microtitre plate were kept at 37◦C for 24 h. Then, DW was used to wash the wells that were stained using 180 µl volume of crystal violet (0.1%). Later, the stained wells were destained using acetic acid and were read using ELISA at 570 nm. As mentioned in the protocol of biofilm construction assay, schemes of biofilm formation, which are non, weak, moderate, and strong biofilm producers, were detected.

Detection MIC of planktonic cells

MIC was detected for planktonic cells using a two-fold broth dilution assay as follows: 100 µl of planktonic cells mixed with 100 µl of serial dilution of antibiotics (As mentioned previously) and poured in 96 wells of microtitre plates. OD was determined for each well at wavelength 570 nm using ELISA after incubation of the microtiter plates at 37◦C for 24 h.

Detection effect of green tea* on *K. pneumonia isolates

Well diffusion test was used to determine the effect of green tea on *K. pneumonia* isolates by making pore within Muller Hinton agar plate swabbed with 25 µl of inoculum, then 25 µl of green tea extract was poured in each well and kept for 24 h at 37 ºC. Recording the result through monitoring the inhibition zone.

Molecular investigation and sequencing

The DNA from three forming and three non-forming biofilm isolates was extracted using a Quick DNA extraction kit. Then, PCR was run using forward and reverse primers with `5CGGTAAAGTTACCGACGTATCTTGTACTG3` and 5`GCTGTTAACCACACCGGTGGT AAC3`, respectively for ***MrkA*** gene amplification. The PCR reaction ran with 25 cycles that include 95 ºC for 30 s, 63 ºC for 30s, and 72 ºC for 90 s for denaturation, annealing, and extension, respectively, while the initial denaturation set up with 95 ºC for 5 min. ABI 3730 DNA Sequencer was used for sequencing the PCR product, and the reaction of sequencing was 4.5 µl for both D. W and Big dye while buffer was 10 µl and 0.5 µl,1µl volumes for PCR product and forward primer, respectively.

Statistical test

The proportion of biofilm-constructing and non-constructing isolates was compared using a chi-squared test. In addition, the different numbers of isolates in different schemes were compared using two-way ANOVA hosted in prism.

Results

The result of the construction of biofilm for 20 isolates revealed that the OD of less than 0.18 refers to a non-producer for biofilm with a percentage of 45%, while OD ranged between 0.18 and 0.36 refers to a weak biofilm producer with a percentage of 30%. However, the moderate biofilm constructer had a percentage of 25% and an OD larger than 0.36 (Table 1). The statistical test for comparison between isolates forming and non-forming biofilm showed that there was no significant difference with P-value P = 0.6645. In addition, a two-way ANOVA test was carried out, and it showed that there were no significant differences among the three categories of biofilm with a P-value equal to 0.9. There was no significant difference in different schemes of biofilm, which may highlight that using biofilm as a virulence factor in isolates under investigation is less crucial in the defense strategies of bacteria.

Table 1: percentage and ODs for isolates non-forming and forming biofilm with moderate and weak schemes

strains	%	OD
non-biofilm former	45	< 0.18
weak biofilm former	30	> 0.18 - < 0.36
moderate biofilm former	25	> 0.36

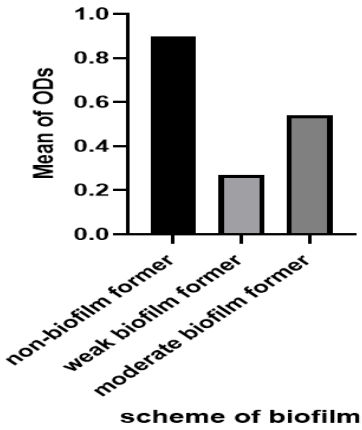

Figure 1: Mean of ODs for different schemes of biofilm formation under study.

The concentration that killed the biofilm-forming bacteria was referred to as MBC, and its result was detected as follows: for Tobramycin, there was no difference in MBC values between isolates forming and non-biofilm forming. However, there were significantly higher MBC values in isolates forming biofilm compared with isolates non-biofilm forming for Ciprofloxacin and Gentamicin. Moreover, for Tobramycin, no difference between MIC and MBC values was detected for isolates of non-forming biofilm and forming biofilm. However, a significant difference between MIC and MBC values was determined for isolates with non-forming biofilm and forming biofilm for Ciprofloxacin and Gentamicin. This indicates the role of biofilm in preventing the antibiotics from penetrating and killing the bacterial cell, while green tea does not have any effect on biofilm (Table 2). We also detected that 60% of isolates were MDR and resisted all antibiotics, including Imipenem, Ceftazidime, Amikacin, and Colistin, and we think this resistance is due to the capacity of these isolates to construct biofilm (Figure 2).

Table 2: MBC and MIC of different antibiotics for isolates forming and non-forming biofilm

	Ciproflo xacin	Ciproflo xacin	Gentamici n	Gentamici n	Tobramyci n	Tobramyci n	Green Tea
isolates	**MIC of cell without biofilm (µg/mL)**	**MBC (Biofilm) (µg/mL)**	**MIC of cell without biofilm (µg/mL)**	**MBC (Biofilm) (µg/mL)**	**MIC of cell without biofilm (µg/mL)**	**MBC (Biofilm) (µg/mL)**	**Appea r or does not appea r growt h**
isolate-5: non-forming biofilm	5	2.5	12.5	12.5	6.25	6.25	growth
isolate-7:non-forming biofilm	5	5	12.5	12.5	6.25	6.25	growth
isolate-12: non-forming biofilm	5	2.5	12.5	12.5	6.25	12.5	growth
Isolate-8: forming biofilm	5	80	12.5	400	6.25	25	growth
Isolate-14: forming biofilm	5	20	12.5	400	6.25	6.25	growth
Isolate-16: forming biofilm	10	10	12.5	50	6.25	6.25	growth

Figure 2: Percentage of MDR isolates that resist Imipenem, Ceftazidime, Amikacin, and Colistin antibiotics used under investigation

The result of the effect of antibiotics and green tea on biofilm formation showed that in the presence of Tobramycin, Gentamicin, and Ciprofloxacin, 55%, 35%, and 40% of isolates inhibit the formation of biofilm, respectively. This indicates that Tobramycin may have a greater effect on biofilm than other antibiotics. Moreover, in the presence of green tea, 40% of isolates inhibit the formation of biofilm, and this may suggest that green tea does not have effect on biofilm formation (Table 3).

For molecular detection of *MrkA* gene in isolates (No: 1, 2, 3) non-forming biofilm and isolates (No: 4, 5, 6) forming biofilm, PCR was carried out, and the result appeared that *MrkA* gene was determined in two biofilm-forming isolates (No: 5, 6), as shown in Figure 3.

Table 3: The effect of different antibiotics and green tea on biofilm maturation.
(↓): inhibition, (↑): induction, (*) No effect

Isolate NO	Tobramycin	Gentamicin	Ciprofloxacin	Green tea
1	*	*	↑	↑
2	*	↑	↑	↑
3	↓	*	↓	↑
4	*	↑	↑	↑
5	↓	↓	↓	*
6	*	↑	*	↑
7	*	↑	*	*
8	↓	↓	↓	↓
9	↓	↓	↓	↓
10	*	↑	↑	↑
11	*	↑	↑	↑
12	↓	↓	↑	↓
13	↓	*	*	↓
14	↓	↓	↓	↓
15	↓	*	↓	↓
16	↓	↓	*	↓
17	↓	↓	↓	↓
18	↓	*	↓	↑
19	*	↑	↑	↑
20	*	↑	*	↑

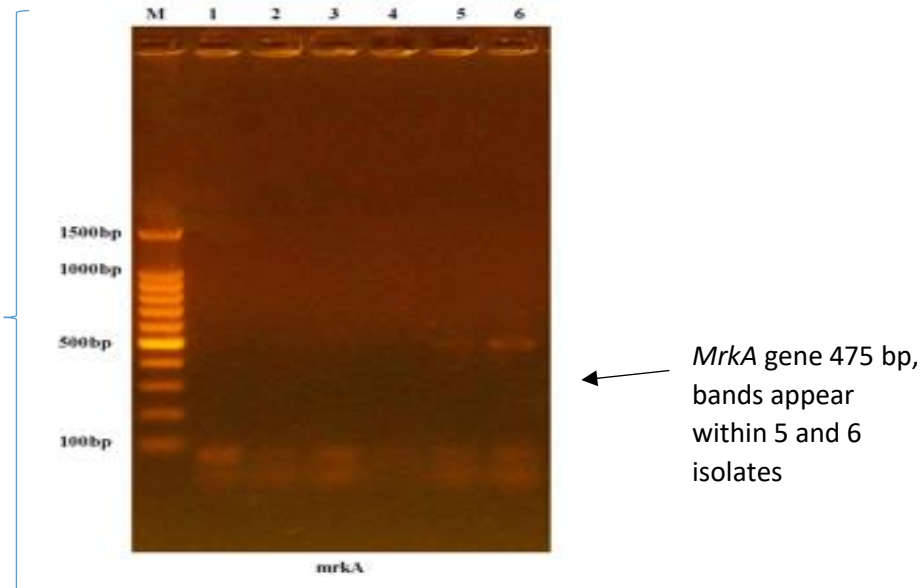

Figure 3: Overall comprehensive gel electrophoresis was carried out on the PCR product of the *MrkA* gene. Line M: Ladder, while Lines 1, 2, 3, 4, 5, 6 are isolates. The size of the amplified band is 475 bp.

Sequencing of *MrkA* gene showed that the sequencing pattern only appeared for isolates No 5 and 6 for *MrkA* gene, and the alignment pattern showed that there was no difference between DNA sequence (Figure 4) and also there was no difference in DNA sequence for aligning DNA sequence of *MrkA* gene for isolate under study with DNA sequence of *MrkA* gene for reference genome (Figure-5).

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           10         20         30         40         50         60
   ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
5  TTACCTGTCT CCGGTAACCC TGACTGAAGT TAAAGCGGCA GCAGCGGATA CTTACCTGAA
6  .......... .......... .......... .......... .......... ..........

           70         80         90        100        110        120
   ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
5  ACCGAAATCT TTCACCATTG ACGTCTCTAA CTGCCAGGCT GCTGATGGCA CTAAACAGGA
6  .......... .......... .......... .......... .......... ..........

          130        140        150        160        170        180
   ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
5  TGACGTAAGC AAACTGGGCG TGAACTGGAC CGGCGGTAAC CTGCTGGCTG GCGCAACCAG
6  .......... .......... .......... .......... .......... ..........

          190        200        210        220        230        240
   ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
5  CAAACAACAG GGCTACCTGG CGAACACCGA AGCTTCCGGT GCTCAGAACA TCCAGCTGGT
6  .......... .......... .......... .......... .......... ..........

          250        260        270        280        290        300
   ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
5  GCTGTCTACT GACAACGCTA CCGCGCTGAC CAACAAAATC ATCCCGGGTG ACAGCACTCA
6  .......... .......... .......... .......... .......... ..........

          310        320        330        340        350        360
   ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
5  GCCGAAAGCG AAAGGCGACG CTTCCGCTGT AGCTGATGGC GCGCGTTTCA CCTACTACGT
6  .......... .......... .......... .......... .......... ..........

          370        380        390        400
   ....|....| ....|....| ....|....| ....|....| ....|...
5  AGGCTACGCC ACCAGCGCGC CGACCACGGT TACCACCGGT GTGGTTAA
6  .......... .......... .......... .......... ........
```

Figure 4: Aligning pattern of DNA sequence of *MrkA* gene for isolate No 5 and 6 that showed no difference in DNA pattern.

Figure 5: Aligning pattern of DNA sequence of *MrkA* gene for isolate under study with DNA sequence of *MrkA* gene for reference genome that showed identity with 100%.

Discussion

Despite the biofilm can be considered a powerful strategy to overcome the problem of displaying the *K. pneumonia* isolates to different antimicrobial agents. In the current study, only 55% of *K. pneumonia* isolates formed biofilm. In controversy, 80% of isolates formed biofilm, as reported by [26]. In addition, 90% of isolates form biofilm, which is resistant to different antimicrobial agents [27]. In the present study, in spite of the percentage of the isolates forming biofilm was low compared with other studies. However, the concentration that killed the bacteria-forming biofilm (MBC) was significantly higher in biofilm-forming isolates compared with isolates non-forming biofilm. Moreover, the MBC mediating biofilm destruction was higher than MIC mediating planktonic cell killing. This result may indicate the biofilm is considered a strong barrier preventing antibiotics from penetrating the bacterial cells. Our result is in agreement with [28], which showed a low percentage of isolates forming biofilm, with 77%; however, most of the isolates forming biofilm were MDR. Antibiotics The effect of different antibiotics on biofilm maturation showed that Tobramycin may have a great effect on biofilm comparable with other types of antibiotics. In addition, green tea showed no significant effect on biofilm formation. However, [29] showed that most *K. pneumonia* isolates were sensitive to carbapenems and quinolones but were resistant to third-generation cephalosporins with 92%. The *MrkA* gene (encoding for Type 1 fimbriae that help in the adhesion of the bacterial cells in epithelial and the construction of biofilm) was detected only in isolates forming biofilm; therefore, we think it has a direct effect on the formation of biofilm. Our result was compatible with [28], which showed that all isolates forming biofilm could harbore the *MrkA* gene. In addition, 90% of isolates harbored the *MrkA* gene, as reported by [19]. Sequencing results for *MrkA* gene showed there was no difference in DNA sequence between isolates No. 5 and 6, and this was because their phenotypic was identical by which both isolates were forming biofilm. Zaborskytė *et al.,* [30] showed that variation in *MrkD* gene contributes to the difference in ability of isolates to form biofilm. In addition, Ochońska *et al.,* [30]showed the genic variation in fimbrial genes may participate in attachment of

bacterial cells to surface effecting biofilm formation. however, it is highly recommended that the number of isolates be increased in future studies on the same topic.

Conclusion

Through the comparison between MBC and MIC for isolates forming and non-forming biofilm, respectively, we concluded that biofilm is a good tool for preventing antimicrobial killing strategy. *MrkA* gene was only detected among isolates forming biofilm with no genic variation through comparison of DNA sequencing.

Conflict of Interest:

The authors state that they have absolutely no conflicts of interest.

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