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# MrKD Gene in Environmental Isolates of Biofilm Producing Klebsiella Pneumoniae

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**Abstract**. Biofilm This concept includes cell aggregates that are not attached to an interface but share traits with biofilms, such as flocs (floating biofilms) and sludge.K. pneumonia is a member of the Enterobactericeae family and is Gram-negative, non-motile, facultatively anaerobic, lactose fermentation, and is present in the environment in places like soil, vegetation, and water. It is also easily isolated from the mucosal surfaces of mammals. K. pneumoniae has at least two variants of type 3 fimbriae, with the most diversified form being the mrkD gene. Materials and Methods: Biochemical and Api20E assays were used to identify K. pneumoniae isolated from different environmental samples in Basra Governorate, Iraq, from 1/11/2021 to 1/1/2022. The Kirby-Bauer assay was performed, and biofilm phenotype formation was evaluated. Finally, the mrkD gene was detected by the PCR method. Results: results showed that the number of K.pneumoniae bacteria was 23 out of 122 samples, and then it was revealed that its ability to form biofilm was used in this study, where the two methods of Congo red were used. The presence of the mrkD gene, which is thought to be responsible for biofilm production, was investigated using a polymerase chain reaction device, and the mrkD gene was found in 34.78% of the samples. Conclusion: This research highlights that the presence of the mrkD gene in K.pneumoniae bacteria, which was isolated from different environmental samples, has a relationship with biofilm formation and shows the extent of biofilm resistance to antibiotics

**Highlights**:

1. Biofilm & K. pneumoniae: Forms biofilms, contains mrkD gene, resists antibiotics.
2. Methods: Identified via biochemical tests, Kirby-Bauer assay, and PCR analysis.
3. Results: 34.78% of isolates carried mrkD gene, linked to biofilm formation.

**Keywords**: Klebsiella pneumoniae, biofilms, mrkD gene.

## Introduction

K. pneumonia is an Enterobactericeae member that is gram-negative, non-motile, facultatively anaerobic, lactose fermentation , and has a conspicuous capsule. It may be found in the environment in places like soil, plants, and water, and it is easily isolated from the mucosal surfaces of mammals. [1]. It is characterized by its ability to form biofilm as a result of exposure to environmental stress such as chemical detergents,

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antibiotics, heavy elements, and pH change [2].The membrane surrounding the microbes is called the extracellular polymeric substance[3] The tissue protects the cells in it and facilitates communication between cells by means of biochemical signals [4] Biofilm is composed of proteins (1-2%), including enzymes, polysaccharides (1-2%), extracellular DNA (1%), and water is more than (97%) than Biofilm, which is The major part responsible for nutrient flow and signal flow within the Biofilm[5] The process of creating a biofilm involves several steps, beginning with attachment to a surface, followed by the development of a micro colony that results in the production of a three-dimensional structure, and eventually maturation and separation. Numerous bacterial species can communicate with one another during biofilm formation thanks to a special process called quorum sensing [6]. The major adhesion (MrkD) of k. pneumoniae type 3 fimbriae has been demonstrated to facilitate binding to collagen in the past. The presence of the MrkD adhesion was not necessary for K. pneumoniae to form effective biofilms on plastic surfaces. However, the presence of the fimbrial shaft on the bacterial surface did help. MrkD, a collagen-binding molecule, seems to be a minor player in the formation of biofilms on these flexible surfaces. Consequently, bacterial binding results in colonisation and the production of biofilms on plastic devices.[7] K. pneumoniae is a serious pathogen due to issues with biofilm formation in this bacterium. The drugs are ineffectual because they have low penetration into the biofilm layer. A bacterial colony known as biofilm promotes surface adherence and aggregation [8]. Bacterial antibiotic resistance in biofilms is caused by a number of methods. First, the EPS that biofilm bacteria secrete serves as a physical/chemical barrier to stop antibodies and many antibiotics from penetrating. Second, embedded biofilm bacteria are often smaller in size, less resistant to antibiotics, and normally do not aggressively divide. Third, incoming antibiotic molecules can be effectively rendered inactive by enzymes that degrade antibiotics, such as -lactamases. Fifth, the antimicrobial drug is rendered inactive in the biofilm's outer layers more quickly than it diffuses. Additionally, biofilms offer a great habitat for the rapid exchange of extra chromosomal DNA that is responsible for antibiotic resistance, virulence factors, and environmental survival abilities. This creates the optimal conditions for the formation of infections with drug resistance[9].

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# Methods

**Samples collection**

(140) samples were collected from different environments: water purification plants, school environments, water tanks, and polluted soil from 1/11/2021 to 1/1/2022, and all samples were cultivated in selective mediums.

**Bacterial Identification**

Most of these isolates came from the environment, and they were recognised as K. pneumoniae using biochemical tests for identifying gut bacteria. Using microscopic techniques to ascertain the morphology of common bacteria, Gram stain, cultural media (pink mucoid colony in MacConkey agar). Various biochemical tests were carried out, including positive catalase test results, negative oxidase test results, glucose gas production, methyl red test results, FeS production, motility, indole production, sodium citrate, and urea [10] Using the Api20E kit (Biomerieux, France), a confirmation diagnosis was made.

**Biofilm formation tests:**

1. Assay of Congo red agar

The biofilm-forming ability of K. pneumoniae was further cross-checked using the CRA plate method)[11]. The bacteria were quadrantly streaked on CRA plates after being created, and they were then incubated at 37 C for 24 hours. The colour variations on each plate after incubation were utilised to identify whether the biofilm colonies were positive or negative. In accordance with CLSI recommendations, biofilm-positive bacteria that produced exopolysaccharide exhibited dark black colonies, while those that did not had red colonies (absence of exopolysaccharide). The low-pink colour colonies reveal the crystalline morphology, whereas the black colour colonies darken to reflect the variation of dry crystalline [12][13] [14]. As shown in figure 1

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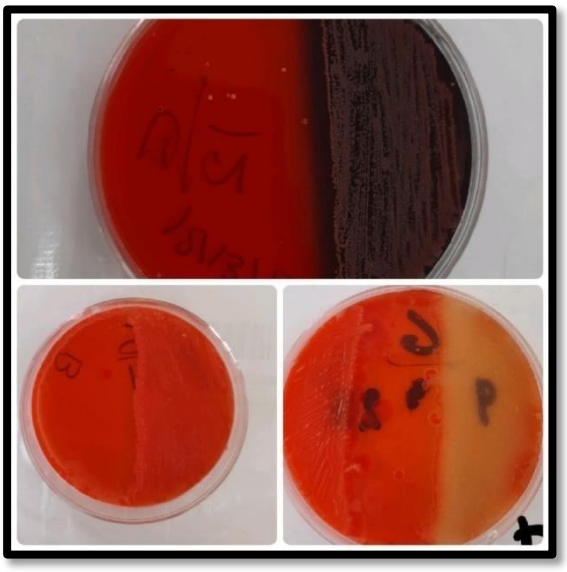

Fig. 1: Qualitative biofilm formation by the CRA method

2. The tube method

Note that the biofilms on the inner walls and the bottom of the tubes are in the form of a violet layer [15]. As shown in figure (2),

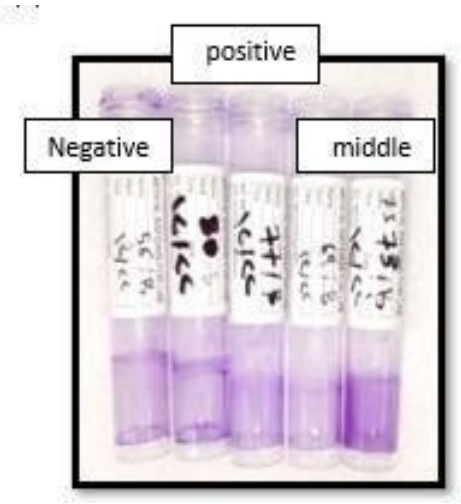


Fig. 2: Qualitative biofilm formation by the Tube method

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**MrkD Detection Gene**

**DNA extraction**

The DNA of the bacterial isolates was extracted using a DNA extraction kit (Geneaid-Taiwan, Presto TM Mini gDNA Bacteria Kit). according to the manufacturer's instructions.

**Detection of adhesion genes using PCR**

PCR was used to identify the chromosomal genes. For PCR reactions, Bioneer master mix PCR (South Korea) is utilized. lists specific primers and PCR products[16]. The PCR products were examined using electrophoresis on a 1 percent agarose gel in 1X TBE buffer. The PCR products were seen under UV light after the gels had been dyed with safe stain.

**Antibiotic resistance**

Using the disc diffusion method on Mueller-Hinton agar, we assessed the susceptibilities to antibiotics of these bacteria. The Clinical and Laboratory Standards Institute recommends using antibiotics to assess these bacteria's resistance [17]

**Statistical analysis**

The statistical programme was used to determine the statistical significance differences between the different variables. SPSS (Statistical Package for Social Science) Ver. 25 In this statistical analysis,

The homogeneity test of samples was performed using Chi-Square Tests at a probability level of 0.05.

## Result and Discussion

The environmental samples, were collected. Then the samples were diagnosed in terms of colony shape and color. Biochemical tests were conducted and the materials and working methods were used according to what was stated in the internationally approved diagnostic systems, as diagnosed by API20E, where (23) samples (18.85%) belonging to the bacteria k. pneumoniae out of (122) were diagnosed. A positive sample Then a biofilm was conducted on it using Congo red agar and the Tube method.

**Klebsiella pneumoniae sensitivity test**

The Kirby-Bauer method was used for all bacterial isolates under study, and it depended on sixteen antibiotics. Biologically from different groups, most of them are

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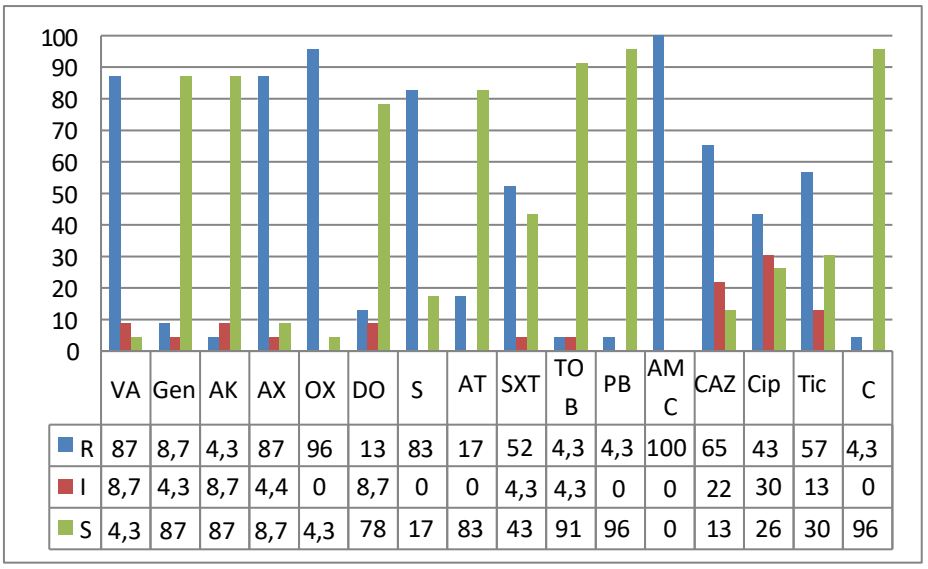
common types that are used to treat different diseases and to know the type of response to the antibiotic. The diameter of the inhibition zone around the anti-disc was measured and the results were compared according to what was mentioned in [17] .The results showed a high resistance to isolates of K. penuminae bacteria isolated from different environments. We find the value of Chi-Square Tests 527.427 with a significance level of 0.05 >0.000 , that is, there are significant differences in the level of effect of antibiotics on K. pneumoniae bacteria. The highest percentage compared to the rest of the antibiotics, followed by The results showed a high resistance of K. pneumoniae bacteria isolated from different environments to AMC, OX, AX, and VA antibiotics, and the resistance percentages were 100%, 96%, 88%, and 88%, respectively. The isolates also showed great sensitivity to C, TOB, and Gen antibiotics. and AK, AT, and DO, respectively, 96%, 92%, 88%, 88%, 84%, 84%, and 80%. As shown in As in the figure (3).




| | VA | Gen | AK | AX | OX | DO | S | AT | SXT | TOB | PB | AMC | CAZ | Cip | Tic | C |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| R | 87 | 8,7 | 4,3 | 87 | 96 | 13 | 83 | 17 | 52 | 4,3 | 4,3 | 100 | 65 | 43 | 57 | 4,3 |
| I | 8,7 | 4,3 | 8,7 | 4,4 | 0 | 8,7 | 0 | 0 | 4,3 | 4,3 | 0 | 0 | 22 | 30 | 13 | 0 |
| S | 4,3 | 87 | 87 | 8,7 | 4,3 | 78 | 17 | 83 | 43 | 91 | 96 | 0 | 13 | 26 | 30 | 96 |

Fig. 3: A diagram showing the preparation of resistant antibiotics by isolates forming biofilms

**PCR detection of the mrkD gene**

Primers were made by an oligo company in South Korea as In this study, 55 isolates out of 140 had the ability to form biofilms, and 23 k.pneumoniae isolates formed biofilms, in addition to eight isolates containing a gene, i.e., (34.78%), indicating a

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correlation between biofilms and a restriction gene. the study. MRKD, as shown in figure (4).


Figure (4): Electrophoresis of PCR amplification of the mrkD gene in K. pneumoniae with an expected product length of 228bp in a (1%) agarose gel. M lane (100bp ladder). Lanes 1, 2, 3, 4, 5, 6, 7, and 8 have positive mrkD genes.

**Discussion**

Biofilms are highly organised mono-or multi-species microbial communities that form either on natural surfaces, such as human organs and other host organisms, or on industrial surfaces such as medical devices, ships, water pipes, and metals used in the oil and gas industry [18]. The importance of biofilms in wastewater treatment[19] Producing biofilm isolates makes it possible to develop bacteria that are more resistant to antibiotics, which leads to treatment failure, rising treatment costs, and a rise in mortality [20] In K. pneumoniae, the genes mrkD adhesions of type 3 fimbriae, which binding to the extracellular matrix, that necessary for biofilm formation. About 34.78% of K. pneumoniae isolates were capable of biofilm formation , And that some of them formed biofilms, despite the absence of the mrkD gene in them, which did not agree with our study in the United States of America, where they proved that the absence of the mrkD gene does not form a biofilm of bacteria. .The reason may be that bacteria have more than one gene to form a biofilm, or the nature of the environment in which they are found is different [7] Our study agreed that the mrkD gene has a role in biofilm

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formation as they demonstrated that biofilm growth by mrkD may be important for the survival of these organisms within the hospital environment[21] Many studies have revealed that type 3 fimbriae (mrkD gene) are important in K. pneumoniae biofilm formation[22] The results of our study showed that there is a high resistance of bacterial isolates to the antibiotics Oxacillin (OX), Amoxillin (AX), Amoxicalar (AMC), and Ciprofloxacin (CIP), respectively (91.82), (91.73), and (92.73), (41%), respectively. Whereas a study in Basrah showed 100% resistance to Amoxillin and resistance to CIP (50%), which is an approach to the results of our study [23](. In another study, the resistance to Amikacin (AK), Gentamycin (Gen), Tobramycin (TOB), and (74.5), (56.6), and (82.9) was shown, respectively, which did not agree with the results of our study, and the reason may be due to the ability of bacteria to produce modified enzymes. For a group of aminoglycids, including the enzymes Acyltrsanferase, Nucleotidytransferase, and phosphtransferase, in addition to reducing the concentration of anti-aminocladic acid inside the cell by reducing the diameter of channels in the outer membrane, While the results of Amoxillin and Ciprofloxacin (84.4 and 46.8), respectively, were in agreement with the results we found[24] A study in Egypt found similar results to our study in the resistance of the antigen (S) streptomycin by 80%. It also agrees with our study on the sensitivity of antibiotics (AK). Amikacin, Chlroamphenicol (C) and Gentamycin (Gen) are 72%, 91%, and 87%, respectively.There is a study in Jordan that did not agree with our study, as the resistance to Ciprofloxacin (CIP), Gentamycin (Gen) and Tobramycin (TOB) was 6.9%, 39.7%, and 77.7%, respectively, and that the anti-Ciprofloxacin (CIP) of the quinolones was due to its resistance as a result of inhibiting the process of entry of the antibiotic into the cell, but in agreement with our study, the resistance to Vancomycin antigen reaches 80% [25]. Because bacteria are becoming more and more resistant to antibiotics [26] .There is a significant rise in antibiotic resistance associated with Beta-lactamasese. One potential explanation for this enhanced resistance is the development of biofilms. The development of biofilms in isolates prevents antibiotics from reaching the bacteria, lengthening the course of treatment by causing an increase in antibiotic resistance. Additionally, the development of multidrug resistance appears to be linked to increased biofilm generation. As a result, more research is being done in Iraq to determine the pattern of antibiotic resistance linked to the development of K. pneumonia biofilm.

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