Detection of fimH, mrkD and mrkC genes in biofilm-forming Klebsiella pneumoniae isolated from different clinical cases

الكشف عن جينات fimH و mrkD وmrkC في بكتريا Klebsiella pneumoniae المكونة للغشاء الحيوي و المعزولة من حالات سريرية مختلفة

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Abstract

Klebsiella pneumoniae is an opportunistic pathogen that forms biofilms on a variety of surfaces. 100 samples were collected from different clinical sources including (burns, wounds, sputum, urine and blood) during the period between 7/30/2024 and 9/30/2024. The aim of this study was to detect the fimbrial genes (fimH, mrkD and mrkC) in K. pneumoniae isolated from different Clinical sources according to their ability to resist antibiotics and produce biofilm. When diagnosed using Vitek 2 technology, fifty of them were identified as K. pneumoniae. The vitek 2 compact system was utilized to evaluate the antibiotic susceptibility of all the isolates. Results revealed a range of resistance percentages, including 90% for(Ampicillin, ceftazidim and cefoxitin) , 80% for cefepime , 62% for ceftriaxone and cefazolin , 64% for ciprofloxaci, 70 % for Trimethoprim/Sulfamethoxazole, 60% for Amikacin and gentamicin , 52 % for ertapenem , 50% for Imipenem and 8% for tigecycline . Biofilm production assay was performed using the 96-well microtiter plate technique. The results showed that 48 isolates (96%) produced biofilm. Genes involved in biofilm formation (fimH, mrkD and mrkC) were detected using PCR method, Eleven

isolates were selected for molecular analysis, all of which were MDR and powerful biofilm makers. The results showed that 11 isolates (100%) possessed the fimH and mrkD genes, and 10 isolates (90.9%) possessed the mrkC gene.

Introduction

Klebsiella pneumoniae is a class of gram-negative bacterium that is ubiquitously found in the environment (such as water, soil, etc.) , or on the surface of mucosa in animals. In humans, K. pneumoniae is concentrated in the gastrointestinal tract, and a few in the nasopharynx, through which the bacteria can enter the blood circulation or other tissues, and then cause infection. In the era of pre-antibiotics, K. pneumoniae was a vital pathogen of community-acquired pneumonia (CAP), especially in diabetics and alcoholics. In the era of antibiotics that followed, it became a major cause of medical-related infections in hospitals[(1)] . The risk groups for K. pneumoniae infections includes newborns, the elderly, and immunocompromised individuals; however, the bacterium is also responsible for an increasing number of community acquired infections[(2,28,29)] . People most at risk for Klebsiella pneumoniae infection are newborns, the elderly, and those with weakened immune systems. Klebsiella bacteria often first reside in the gastrointestinal tract and can be spread between patients via the hands of hospital staff [(3)] . Klebsiella pneumoniae has received significant and growing attention worldwide as an infectious microorganism, due to the significant increase in the number of severe Klebsiella pneumoniae infections, antibiotic resistance, and the significant difficulty in finding effective treatments. Klebsiella pneumoniae is now the second most common cause of Gram-negative bacteremia and a major cause of hospital-acquired infections, particularly in immunocompromised patients[(4)] . Over the past few years, we have witnessed an alarming increase in the development of strains derived from "classic" bacteria that are resistant to a wide range of antibiotics. As a result of this resistance, some simple infections, such as urinary tract infections, have become untreatable, while more serious infections, such as pneumonia and bacteremia, have become increasingly life-threatening [(5)] .

Biofilms are surface-associated communities of microorganisms. Biofilm-associated bacteria are protected from host defenses and antibiotics and are the cause of many infections. Klebsiella pneumoniae is primarily a hospital-acquired bacterial pathogen that causes pneumonia, urinary tract infections and septicemia. Its success is related to its ability to form biofilms on medical devices, such as catheters. In K. pneumoniae, biofilm formation is mediated by type 3 fimbriae –

hair-like, protein appendages extending out from the cell surface that adhere to surfaces[18]. The ability of bacteria to adhere to tissue surfaces in the host is an important step in the development of infection. Most clinical K. pneumoniae isolates express two types of fimbrial adhesins, type 1 and type 3 fimbriae [11] . Bacterial adherence to host mucosal surfaces is often an important first step in the infection process. This is especially true in the case of urinary tract infections (UTIs) [6] . The most common type of adhesive organelle in the Enterobacteriaceae is the type 1 fimbria,The corresponding structures of Klebsiella pneumoniae are similar to those of E. coli with regard to genetic composition and regulation [7] . Type 1 fimbriae are composed primarily of the structural subunit FimA, with minor amounts of three ancillary subunits, FimF, FimG, and the mannose-specific adhesin FimH. FimH is one of the genes in K. pneumoniae that encodes for virulence factors responsible for the majority of damage caused by bacteria[17] . The FimH adhesin is an allosteric protein that mediates the catch bond mechanism of adhesion where the binding is increased under increased shear stress [8] . K. pneumoniae possesses a number of known virulence factors, including a pronounced capsule, type 3 fimbriae, and type 1 fimbriae [9] . Type 1 fimbriae produced by K. pneumoniae are described as functionally and structurally similar to type 1 fimbriae from E. coli [10] . Compared to type 1 fimbriae, type 3 fimbriae are thin (4 to 5 nm in diameter) nonchanneled fimbriae [12] . They are characterized by their ability to agglutinate tannic acid treated, but not native, erythrocytes in a mannose-resistant manner; also referred to as mannose-resistant Klebsiella-like hemagglutination. In addition to Klebsiella species, type 3 fimbriae are common in Enterobacter, Serratia, Proteus, and Providencia isolates [13]. Type 3 fimbriae belong to the chaperone-usher class fimbriae and are encoded by the mrk gene cluster, which includes the mrkD encoding the fimbrial adhesin responsible for mannose-resistant Klebsiella-like hemagglutination [14]. The MrkD adhesin has been shown to mediate adhesion to collagen structures [15] . however, the exact identity of the MrkD receptor remains elusive. In vitro studies have revealed that type 3 fimbriae mediate adhesion to different structures in human kidney and lung tissue and epithelial cells from human urine sediments, as well as in endothelial and bladder epithelial cell lines[16] . The aim of this study was to detect the fimbrial genes (fimH, mrkD and mrkC) in K. pneumoniae isolated from different Clinical sources according to their ability to resist antibiotics and produce biofilm.

Materials and Methods

Collection of Samples

100 samples were collected from different clinical sources including (burns, wounds, sputum, urine and blood) from Al-Kadhimiya Hospital, Martyr Ghazi Al-Hariri Hospital, Imam Ali Hospital, Yarmouk Hospital, Ibn Al-Balady Hospital, Burns Hospital and Children's Hospital during the period between 7/30/2024 and 9/30/2024. All of these specimens were grown overnight at37^{o}C on MacConkey agar , Blood agar, Eosin Methylene Blue agar and CHROMagar Orientation (Himedia/ India) (19,27,26) . Then, the pure colonies were selected and identified by several biochemical assays, including oxidase, catalase (32, 33).Later, the isolates were identified using biochemical tests in vitek- 2 system(25)

Antimicrobial sensitivity test

The susceptibility test for 50 K. pneumoniae isolates to Fifteen antibiotics belong to (seven groups) of antibiotics was determined by using Vitek -2 compact system. The antibiotics utilized in this study are shown in table (1)

Table (1) Antibiotics utilized in this study

ANTIBIOTIC GROUP	ANTIBIOTIC
Aminoglycosides	Gentamycin, Amikacin
Cephalosporins	Cefazolin, cefoxitin,ceftazidim ceftriaxone, cefepime
Carbapenems	Imipenem , ertapenem
Tetracyccline	tigecycline
Fluoroquinolones	ciprofloxacin ,levofloxacin
Penicillin	Ampicillin piperacilin/tazobactam
Trimethoprim Sulfamethoxazole	trimethoprime/sulfamethoxazole

Biofilm formation assay

Quantitative assessment of biofilm formation was performed by colorimetric microtiter panel assay as previously described by (20 ,29) . A K. pneumoniae sample was cultured for 24 hours after which the biofilm turbidity was adjusted and compared to a McFarland 1 tube. Suspensions were diluted 1:100 in 200 µL

of Brain-heart infusion broth, They were then transferred to sterile 96-well flat-bottom polystyrene microplates. The plates were gently washed three times with sterile phosphate-buffered saline (PBS) after 24 hours of incubation at 37°C. The adherent biofilms were fixed with 99% methanol for 15 minutes, and the solutions were then removed , The plate was air-dried. Biofilms were stained with 200 µL of 1% crystal violet for 5 min at room temperature, then rinsed with water and allowed to dry. The biofilm stain in each well was removed by treating it with 200 µL of 95% ethanol for 30 min. The absorbance was measured at 630 nm using an ELISA reader. After comparing the optical density (OD) of the biofilm with that of the control sample, based on the readings, the isolates were classified as weak (OD control <OD isolate ≤2×OD control), moderate (OD control <OD isolate ≤4×OD control), and strong (OD isolate >4×OD control).

Extraction of Genomic DNA

DNA was extracted from overnight K. pneumoniae colonies that grew on MacConkey agar According to ABIOpure instructions .

Detection of fimH & mrkD & mrkC genes in K.pneumoniae isolates

FimH and mrkD and mrkC genes were detected by PCR for 11 isolates using specific primers as shown in table (2). The PCR mixture set up in 20 µl total volume consisting of 10 µl FIREPol® Master Mix and 1 µl of each primer and 2 µl for DNA's template Then complete the volume to 20 microliters of distilled water does not contain cutting enzymes and ions. The thermal program was optimized and executed on a master cycler (Eppendorf, Germany) as shown in the table below (3).

Table (2) Specific primers for fimH ,mrkD and mrkC genes

Primer Name	Seq.	Annealing Temp. (°C	Product Size (bp)	Reference
fimH-F	5`- TGCTGCTGGGCTGGTCGATG-3`	58	550	(Alwan et al.,2024)
fimH-R	5`- GGGAGGGTGACGGTGACATC-3`			
MrkD-F	5`- GTCTTTTCGTCCCGGGTATATAA		244	(Bakhtiari et

	C-3`			al.,2021)
MrkD-R	5`-CCACATCGACATTCATATTTTTCC-3`			
MrkC-F	5`GAATTGTTGTAGCTGACCTGA-3		1155	(Muhsin et al.,2022)
MrkC-R	5`-TCAACGCCTGAAAAACTATGT-3`			

Table(3) Thermal programme utilized in this study

Steps	°C	m:s	Cycle
Initial Denaturation	95	05:00	1
Denaturation	95	00:30	30
Annealing	58	00:30	
Extension	72	00:30	
Final extension	72	07:00	1
Hold	10	10:00	

Results and Discussion

One hundred sample that collected from different sources including:1-blood(24 sample 24%) 2-urine (45 sample 45%) 3-sputum (15 sample 15%) 4-wound (16 sample 16%)were utilized to isolate Klebsiella pneumoniae. Fifty-five isolates which gave pinkmucoid colonies on MacConkey agar were identified by Vitek -2 compact device, the results revealed that(50) of the bacterial isolates were Klebsiella pneumoniae . everal studies revealed that the most common site of K. pneumoniae infections is UTIs[30] , followed by bloodstream infections, pneumonia, and burns and wounds infections [21] . Fifty isolates of Klebsiella pneumoniae were tested for 7 groups of antibiotics using Vitek -2 compact system, and it was found that the highest Percent of bacterial resistance for Penicillin (90%) , and Carbapenem(25%) , Aminoglycoside (54%), Tetracyccline (4%), Cephalosporins (80%) , Fluoroquinolones (72%), Trimethoprim/

Sulfamethoxazole (50%). During this time, K. pneumoniae acquired antibiotic resistance mechanisms, including drug-modifying enzymes with diverse activities (adenylation, acetylation or phosphorylation). Within ten years, all types of plasmid-mediated resistance genes of aac, aph, and ant gene families in K. pneumoniae were found. The reduced use of aminoglycoside slowed the evolution of the novel resistant genes until the 16SrRNA methylase occurred, which was belonged to the armA gene family, encoding an enzyme that blocks the binding of aminoglycoside antibiotics to the 16SrRNA [(31)]

The results of the investigation of the ability of Klebsiella isolates to produce biofilm using the Microtiter Plate (MTP) method according to the optical absorption of the ELISA device. 47 0f the isolates studied produced biofilms, i.e., 94 %, but with varying intensity when compared to the reading rate of the negative control , The result was 20 isolates were strong (40%), 25 isolates were medium (50%), 3 isolates were weak (6%), and 2 isolates were non-forming (4%).

Genotypic screening revealed that fimH and mrkD were detected in 11 (100%) of K. pneumoniae isolates with a PCR product 550bp for fimH and 244 bp for mrkD , whereas mrkC was found only in 10 (90.9%) of isolates with a PCR product of 1155 bp, as shown in Figures-(1 , 2 and 3)

This is close to what was reached by (22) whose results showed that 98% of the isolates possessed the mrkD gene, And with (23) the results showed that 100% of the isolates contain the fimH gene , And with (24) the results showed that 87% of the isolates contain the mrkC gene

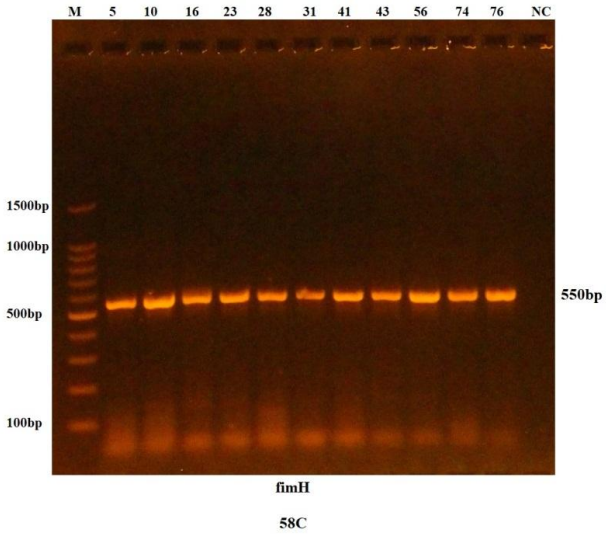

Figure (1): Results of the amplification of FimH gene of Klebsiella pneumoniae samples species were fractionated on 1.5% agarose gel electrophoresis stained with Eth.Br. M: 100bp ladder marker. Lanes 5-76 resemble 550 bp PCR products, NC: negative control.

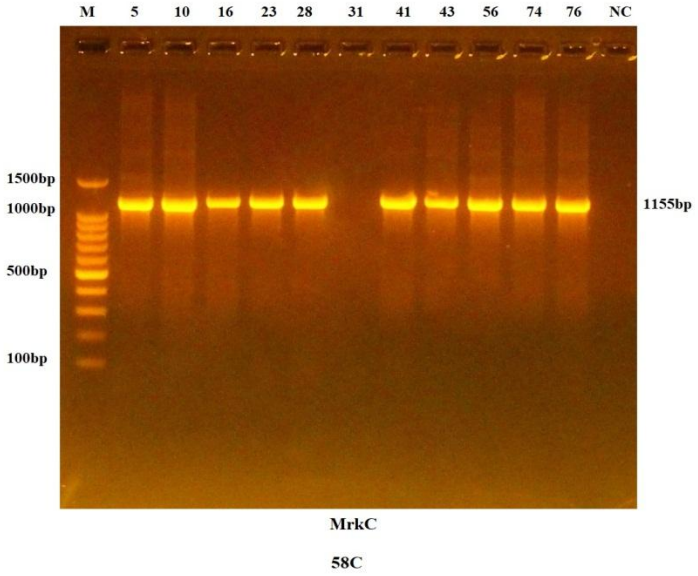

Figure (2): Results of the amplification of MrkC gene of Klebsiella pneumoniae samples species were fractionated on 1.5% agarose gel electrophoresis stained with Eth.Br. M: 100bp ladder marker. Lanes 5-76 resemble 1155 bp PCR products, NC: negative control..

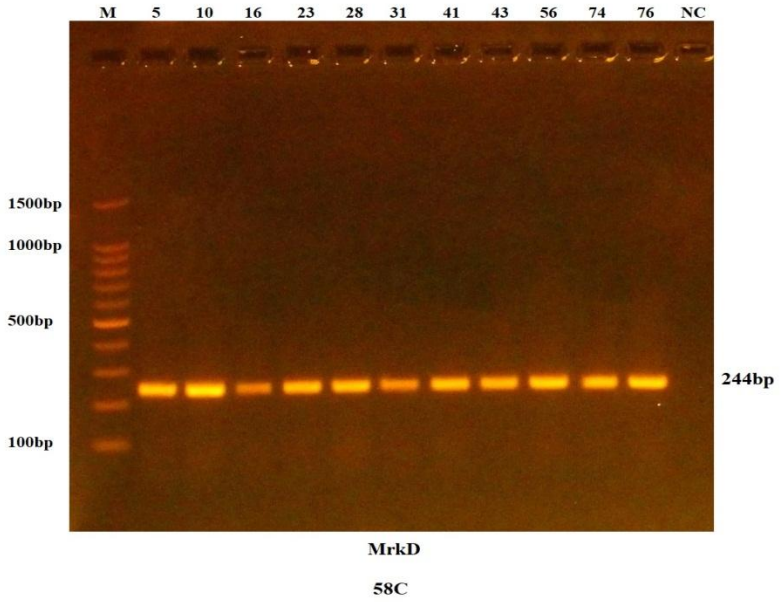

Figure (3): Results of the amplification of MrkD gene of Klebsiella pneumoniae samples species were fractionated on 1.5% agarose gel electrophoresis stained with Eth.Br. M: 100bp ladder marker. Lanes 5-76 resemble 244 bp PCR products, NC: negative control.

Conclusion

We conclude from the results of this study that 48 (96%) clinical isolates of K. pneumoniae were biofilm producers, with 25 isolates (50%) being moderately strong in biofilm production, 20 isolates (40%) being strong, and 3 isolates being weak, with the majority being MDR. R and having genes responsible for adhesion in percentage %100 for fimH and mrkD , and %90.9 for mrkC.

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