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DETECTION OF GENES RESPONSIBLE FOR BIOFILM FORMATION IN DIFFERENT BACTERIA CAUSING CATHETER ASSOCIATED URINARY TRACT INFECTION.

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ABSTRACT

This study aimed to detect biofilm forming bacteria associated with urinary catheters and to detect genes responsible for biofilm formation. The biofilm formed by bacteria isolated from urinary catheters has been determined by safranine staining. Furthermore, the genes responsible for biofilm formation have been detected by using the PCR technique. A total of 119/150 (79.3%) of clinical isolates obtained from urinary catheters were positive for biofilm formation. The two genes responsible for the biofilm formation, type 3fimbriae *(mrkABCDF)* gene and type 1 pilus *(fimK)* gene were detected in the PCR of *E.coli* and *Klebsiella* isolates, respectively. The two copies of mrpA genes (mr1 and mr2) which control the expression of MR/P fimbriae in Proteus species were detected in the PCR products on agarose gel at 1000 bp for mr1 and at 850 bp for mr2 genes, respectively. In *Pseudomonas tox A* gene was detected at 200 bp. In addition, *ica A* gene (190 bp) and *ica D* gene (200 bp) were detected on agarose gel in PCR products of *S. epidermidis* and *S. aureus* isolates at 190 bp and 200bp, respectively.

INTRODUCTION

Many bacteria are able to adhere to living and nonliving surfaces, including those of indwelling medical devices, and form biofilms made up of extracellular polymers. In this state, the microorganisms are highly resistant to antimicrobial treatment and are tenaciously bound to the surface. To better understand and control biofilms on indwelling medical devices, researchers should develop reliable sampling and measurement techniques, investigate the role of biofilms in antimicrobial drug resistance, and establish the link between biofilm formation and patient infection [1].

Microbial biofilms developed when microorganisms irreversibly adhere to the polymeric surface and produce extracellular substances that facilitate adhesion and provide a structural matrix. This surface may be inert, nonliving material or living tissue. Biofilm-associated microorganisms behave differently from planktonic (freely suspended) organisms with respect to growth rates and ability to resist antimicrobial treatments and therefore pose a public health problem. Microbial biofilms were developed on or within indwelling medical devices (e.g. urinary catheter) [1].

E.coli is a major cause of catheter associated urinary tract infections (CAUTI) and several surface factors that contribute to biofilm growth by *E. coli* have been characterized. Type 1 and type 3 fimbriae expression is associated with persistence in the long term catheterized urinary tract [2]. Flagella are motility organelles that play a role in the initial adhesion phase of biofilm formation [3]. Type 3fimbriae is produced by many members of the *enterobacteriaceae* family that are associated with opportunistic infection [2].

Biofilm growth is mediated by type 3 fimbriae may be important for the survival of these organisms on the surfaces of urinary catheters. The role of types 3 fimbriae in biofilm formation was confirmed by the complementation of each of these mutants with a plasmid containing the *mrk* genes [3].

K. pneumoniae is the second most common cause of gram negative UTI. A novel regulatory *(fimk)* gene has been identified in *K. pneumoniae*. This regulatory element can control the expression of type 1 pilus which aid in the colonization and infection of *K. pneumoniae* to the urinary tract.The effects of *fimk* gene on type 1 pilus expression which give the ability of *Klebsiella pneumoniae* to form type 1- dependent biofilms. The *fimk* gene may act exclusively on the expression of type 1 pilus. In addition ,the *fimk* gene may also affect the expression of other adhesins or other virulence factors in *K. pneumoniae* that might play a role in UTI [4].

The role of (MR/P) fimbriae of *P. mirabilis in* colonization of UT has been confirm. The genes encoding (MR/P) fimbriae is located in complex fimbrial operon [5]. *P. mirabilis* mutants have been generated to explore the role of (MR/P) fimbriae of *P. mirabilis in* UTI [6]. The importance of *mrpA* genes in the expression of MR/P fimbriae in *P. mirabilis* in UTI has been elucidated.

P. aeruginosa isolates recovered from patients suffering from urinary tract infections produced significant level of *exotoxin A (tox A)*. Equally, up regulation of *exotoxin A* may be observed within biofilms due to the action of quorum sensing [7]. The *exotoxin A* of *Pseudomonas aeruginosa* may play a significant role as a virulence factor in CAUTI [8].

Among the *ica* genes, *icaA* and *icaD* have been reported to play a significant role in biofilm

formation in *S. aureus* and *S. epidermidis*. The *icaA* gene encodes N-acetyl glucosaminyl transferase, the enzyme involved in the synthesis of N-acetyl glucosamine oligomers from UDP-N-acetyl glucosamine. Furthermore, *icaD* gene has been reported to play a role in the maximal expression of N acetyl glucosaminyl transferase, leading to the phenotypic expression of capsular polysaccharide [9].

The aim of this study was to detect the biofilm-forming capacity of microorganisms isolated from the urinary catheterized patients. In addition, the genes responsible for the formation of biofilms were examined in the PCR products of such isolates.

EXPERIMENTAL

Chemicals

Safranine was supplied by Oxford Laboratory. Ethidium bromide and ethylene diamine tetra acetic acid disodium salt (EDTA) were supplied by Merck, Germany. Tris- HCL, Triton X-100, Lysozyme, Chlorhexidine digluconate solution 20%, Tetra methyl p- phenylene diamine Hcl and Agarose DNA grade were supplied from Sigma-Aldrich, Company. Electrophoresis buffer TAE (50x) was obtained from Ferments Life Science, Germany.

Bacterial isolates

Clinical isolates of *E. coli*, klebsiella spp, proteus spp, *Pseudomonas aeruginosa*, *S. epidermidis* and *S. aureus* were isolated from urinary catheters obtained from patients attended the Urology Department at Belqas Hospital. The isolates were identified by Gram staining, testing on TSI, IMViC, motility, swarming test and mannitol salt agar.

Detection of biofilm formation

Detection of slime production was performed according to Kadry *et al.* [10]. Briefly rounded glass tubes were used to assay for adherence. The growth cultures of nutrient broth were incubated at 37°C for 48h, the contents of tubes were decanted, and the tubes were stained with safranine to demonstrate adherent growth. Presence of adherent growth was taken as evidence of slim formation and hence biofilm formation.

DNA extraction

The low copy plasmid DNA from *Escherichia coli* and *Klebsiella pneumoniae* were obtained according to the manufacturer's procedure of Bioline Kits. Genomic DNA purification from *Proteus mirabilis* and Gram positive bacteria (*S. epidermidis* and *S. aureus)* were performed according to the manufacturer's procedure of Fermentas Kits. In addition, the extraction of mRNA from *Pseudomonas aeruginosa* was performed according to the manufacturer's procedure of Axygen Biosciences Kits.

Polymerase Chain Reaction

Polymerase chain reaction (PCR) components

PCR Master Mix (2x) was obtained from AB gene UK and composed of Dream Taq DNA polymerase supplied in reaction buffer, dNRPs and 4 μm $Mgcl_2$. Gene Ruler 100 bp plus DNA ladder obtained from Thermo Scientific Inc, U.S.A. The primers for PCR reaction (Table 1) were designed by Operon Biotechnologies GmbH Biocompus Cologne, Germany.

Genomic DNA purification kit was obtained from Fermentas Life Science and the Mini kit for plasmid isolation was obtained from Bioline meridicin Life Science Company. RNA purification kit was obtained from Axygen Biosciences.

Table (1): Primers used in PCR amplifications.

M.O	Primer	Sequence (5-3)	PCR product size
Escherichia coli	871 F	CGCGCGATATCGCAGCATAACCGAAACAAATG	1000- bp
	872 R	CCGGGATATCTAAATTTTCTGCGGCAAACC	
Klebsiella pneumonia	*fim KF1*	CGGTAACGCGGCCGCGACTATCCGGAAACGATCACC	1000- bp
	fim KR2	AACAAGCTTAGACGATCCGATGACTCAC	
Proteus mirabilis	*5mr1F*	AAACGCCTGACATAGAGTAT	991 – bp
	3mr1R	CATCAATGATGGATCCAACAA	
	5mr2F	CAAATTAGGATCCCCAAGTGCT	882- bp
	3mr2R	CCGAATAGCATGCTTTTTTGTA	
Pseudomonas aeurginosa	*toxAF*	GGAGCGCAACTATCCCACT	200-bp
	toxAR	TGGTAGCCGACGAACACATA	
Staphylococcus aureus and *S epidermidis*	*icaAF*	TCTCTTGCAGGAGCAATCAA	188-bp
	icaAR	TCAGGCACTAACATCCAGCA	
	ica DF	ATGGTCAAGCCCAGACAGAG	198- bp
	ica DR	CGTGTTTTCAACATTTAATGCAA	

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Electrophoresis of genomic and plasmid DNA

The molten agarose (1%) in TAE buffer was poured in the running chamber with appropriate comb and left to solidify. The comb was removed after flooding the gel with TAE buffer. About 10μL of 10 mg/mL ethidium bromide (EtBr) solution was added for DNA staining. The plasmid DNA or genomic DNA (15μL) was mixed with 2μL of 10x loading buffer and each DNA sample was loaded carefully in separate well using micropipette. The genomic DNA and plasmid DNA were separated according to their molecular size by electrophoresis at 4v/cm. The electrophoretic power was shut off after the bromophenol blue band reached to nearly the end of the gel length. The gel was visualized on UV transilluminator and photographed.

Screening for different genes

Type 3 fimbrial gene *(mrk)* located in plasmid produced by *Escherichia coli* [11] and type 1 pilus *(fimk)* gene from plasmid in *Klebsiella pneumoniae* [4] were screened according to the following protocol: Each PCR mixture contained 25μL of PCR master mix 2x, 2.0μL of forward primer, 2.0μL of reverse primer, 10.0μL of DNA template and nuclease free water to 50μL. The cycle used was 95°C for 3 min, then 35 cycle of 95°C for 1 min, 55°C for 1 min and 72°C for 30 seconds. The PCR products were separated on 1.5% agarose gel, stained with ethidium bromide, visualized by UV transilluminator and photographed. DNA size marker was used for size determination.

A modified technique was used for screening for (MR/P) fimbriae in genomic DNA produced by *Proteus mirabilis* [12] where, each PCR mixture contained 25μL of PCR master mix 2x, 2.0 of forward primer, 2.0 μL of reverse primer, 10.0 μL of DNA template and nuclease free water to 50μL. The cycle used was 94°C for 3 min. 30 cycle of 94°C 1 min, 52°C for 1 min and 72 °C for 1 min and a final cycle of 72°C for 5 min. The PCR products were separated by electrophoresis in 2% agarose gel, stained with ethidium bromide, visualized by UV transilluminator and photographed. DNA size marker was used for size determination.

Exotoxin A gene of *Pseudomonas aeruginosa* was screened according to the following procedure [8]. Each PCR mixture contained 25μL of PCR Reddy Mix (2x), 1.0μL of forward primer, 1.0μL of reverse primer, 2.5μL of RT Enhancer, 5.0μL of template RNA and nuclease free water to 50μL. Reaction condition for *P. aeruginosa toxA* amplification consisted of heating at 95°C for 5 min. followed by 40 cycles of 95°C for 1 min, 60°C for 1 min and 72°C for 30s. The PCR products were separated by electrophoresis in a 2% a garose gel, stained with ethidium bromide, visualized by UV transilluminator and photographed. PCR marker was used for size determination.

The *icaA* and *icaD* genes of *S epidermidis* and *S. aureus* were screened by PCR [13]. Each PCR mixture contained 25μL of PCR master mix 2x, 2.0 μL of forward primer, 2.0 μL of reverse primer, 10.0 μL of DNA Template and nuclease free water to 50μL. The cycle used was 94°C for 3min, followed by 30 cycles at 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds and 72°C for 1 min. The PCR products were separated by electrophoresis in 2% a garose gel, stained with ethidium bromide and visualized by UV transilluminator and photographed. DNA size marker was used for size determination.

Electrophoresis of genomic and plasmid DNA

The molten agarose (1%) in TAE buffer was poured in the running chamber with appropriate comb and left to solidify. The comb was removed after flooding the gel with TAE buffer. About 10μL of 10 mg/mL ethidium bromide (EtBr) solution was added for DNA staining. The plasmid DNA or genomic DNA (15μL) was mixed with 2μL of 10x loading buffer and each DNA sample was loaded carefully in separate well using micropipette. The genomic DNA and plasmid DNA were separated according to their molecular size by electrophoresis at 4v/cm. The electrophoretic power was shut off after the bromophenol blue band reached to nearly the end of the gel length. The gel was visualized on UV transilluminator and photographed.

RESULTS

The degree of slime production and biofilm formation was graded qualitatively and summarized in **figure (1) and table (2)**.

Fig. (1): Biofilm and and growth adherence after staining with safranin. 0 = negative producer, 1=weak producer, 2 =moderate producer, 3= strong producer.

Table (2): Degree and number of biofilm forming clinical isolates.

Microorganism	Total number	Degree and number of Biofilm forming isolates			
		Non	Weak	Moderate	Strong
E. coli	40	13	8	7	12
Klebsiella	28	8	5	7	8
Proteus	20	-	7	7	6
Pseudomonas	22	-	6	8	8
S. epidermdis	28	6	14	4	4
S. aureus	12	4	2	3	3

As table (2) shows, 119 isolates (79.3%) out of 150 were biofilm producers. Among the Gram negative bacteria, 27(67.5%) out of 40 were slime producers at different degrees in *E.coli* isolates .In addition 20 (71.4%) out of 28 *Klebsiella* isolates were slime producers at different levels. However, all *Proteus* (20) and *Pseudomonas* (22) isolates were forming biofilm at different degrees.

In Gram positive bacteria, 22 (78.6%) of *S. epidermidis* isolates were forming biofilm while 8(66.7%) of *S.aureus* isolates were forming biofilm.

Figures (2-8) represented the detection of genes responsible for biofilm formation in major groups of urinary pathogens.

As shown in figure (2), type 3 fimbrial gene (*mrkABCDF*) was detected on agarose gel at approximately 1000 bp from PCR products of *E.coli* isolates.

The *fimk* genes among *klebsiella* isolates were detected in PCR products on agarose gel at approximately 1000 bp (Figure 3).

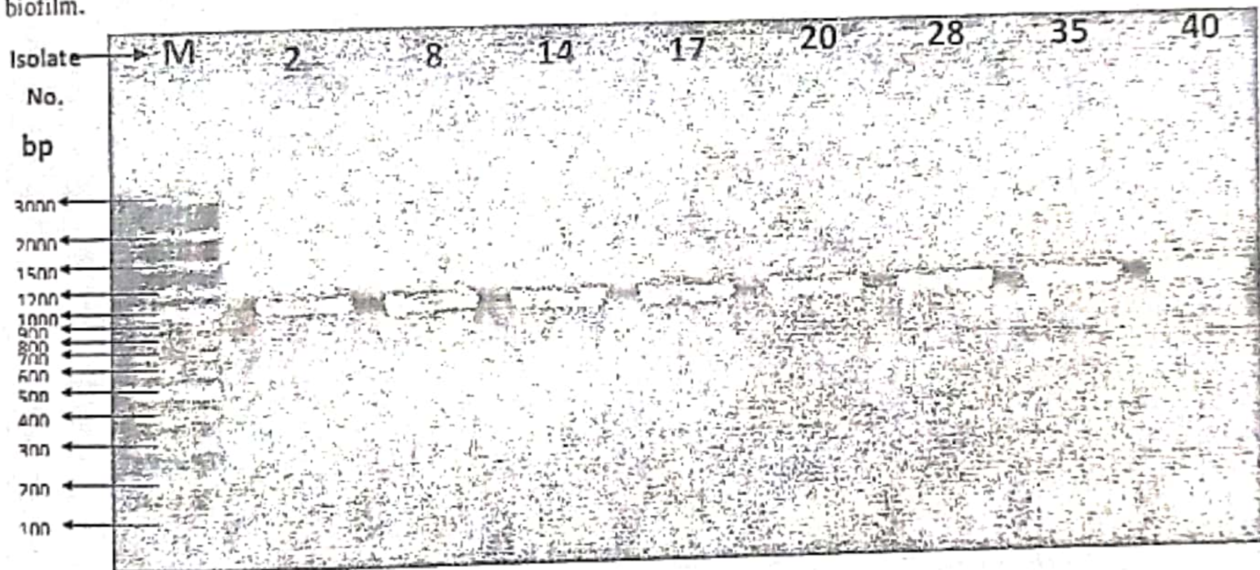

Fig. (2): Detection of type 3 fimbrial gene in PCR products of some biofilm forming *E. coli* isolates.

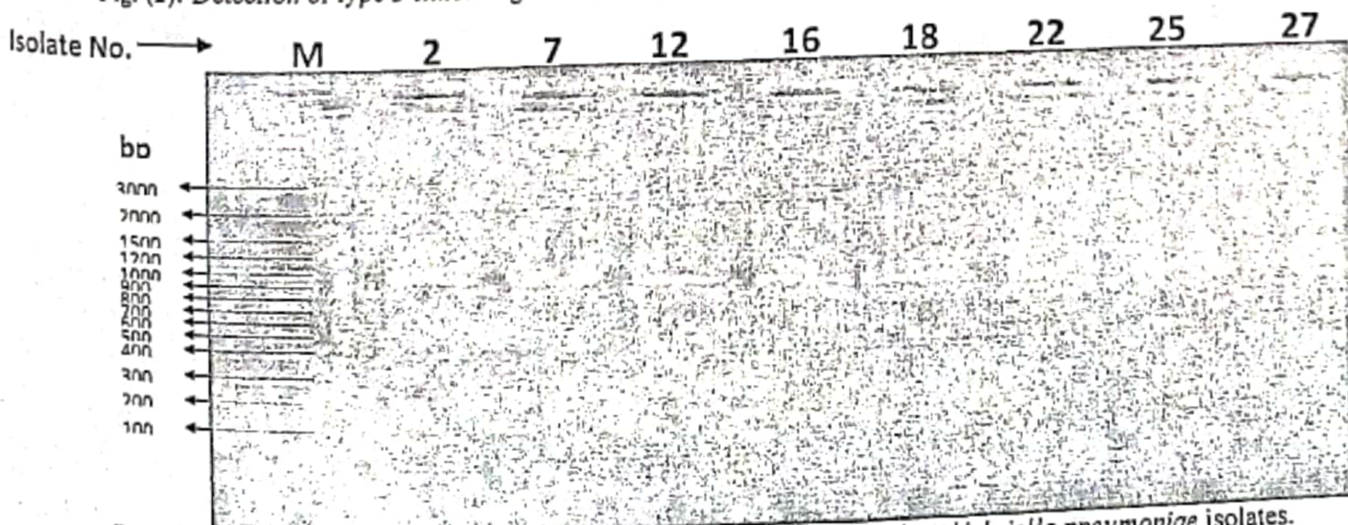

Fig. (3): Detection of *fimk* gene in PCR products of some biofilm forming *klebsiella pneumoniae* isolates.

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The first *mrpA* genes controlling the(MR\P) fimbriae in genomes of *Proteus mirabilis* have been amplified by mr1 oligonucleotides and detected in PCR at approximately 991 bp (figure 4), while the second *mrpA* gene has been detected at approximately 882 bp by using mr2 oligonucleotides (figure 5).

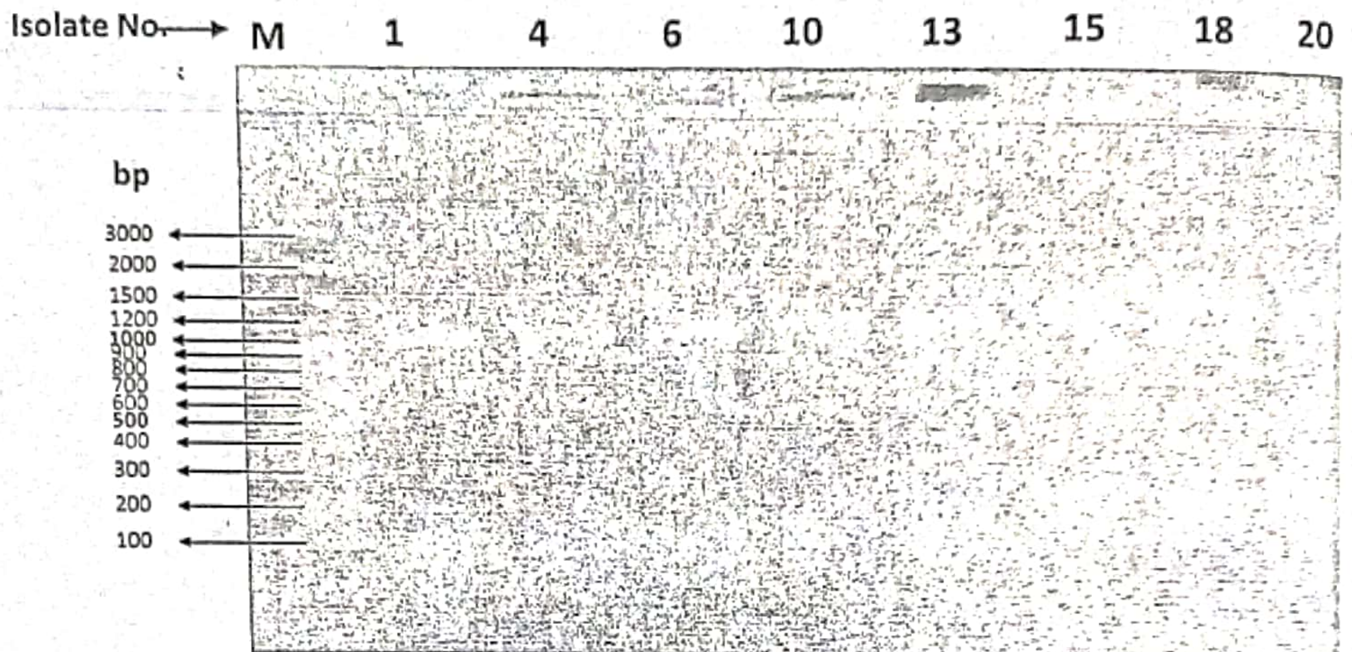

Fig. (4): Detection of *mrp* gene in PCR products of some biofilm forming *P. mirabilis* isolates (amplified by mr1 oligonucleotides).

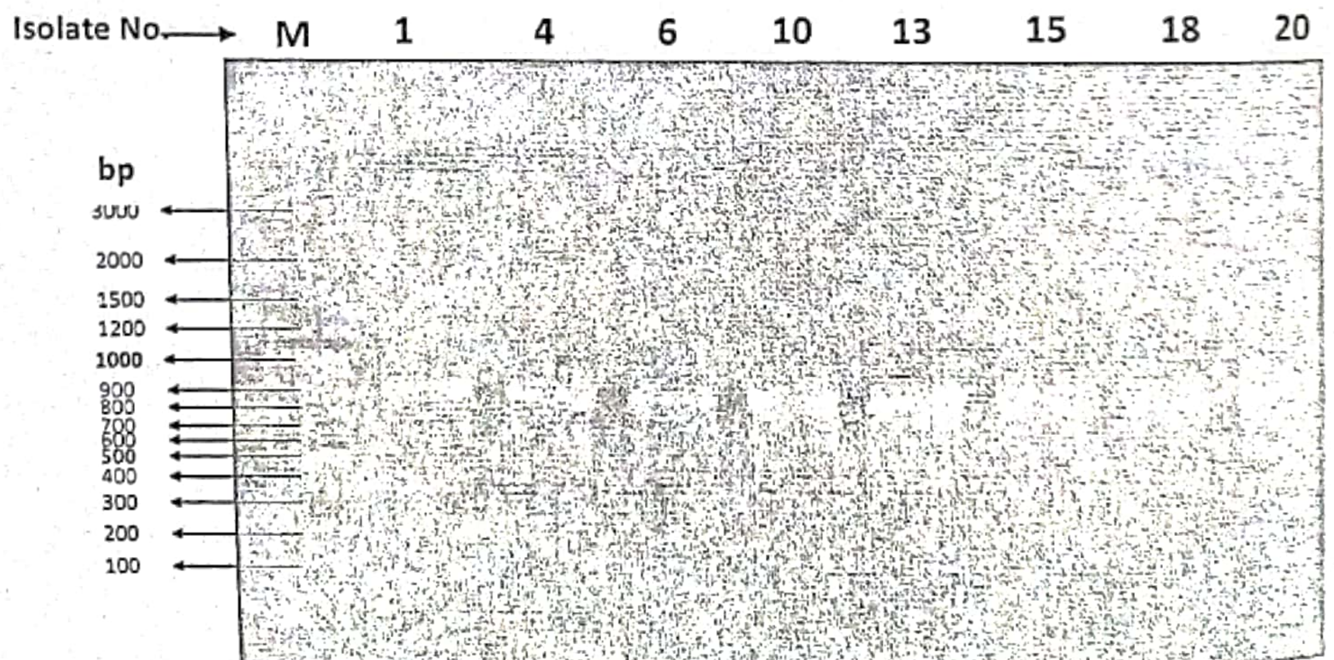

Fig. (5): Detection of *mrp* gene in PCR products of some biofilm forming *P. mirabilis* isolates (amplified by mr2 oligonucleotides).

The analysis of PCR products of *Pseudomonas aeruginosa* isolates harboring the *exotoxin A* gene (*tox A*) revealed that, bands equal to approximately 200 bp have been detected and matched to the size of *tox A* gene responsible for biofilm formation (figure 6).

The screening of *icaA* and *icaD* genes responsible for biofilm formation among the *staphylococcus epidermidis* and *staphylococcus aureus* isolates was summarized in figures (7) and (8). The *icaA* gene was detected in PCR products of *S. epidermidis and S. aureus* at approximately 190 bp (Figure7). In addition, a band measured at 200 bp matched to *icaD* gene was detected in the PCR products of *S. epidermidis and S. aureus* isolates (figure 8).

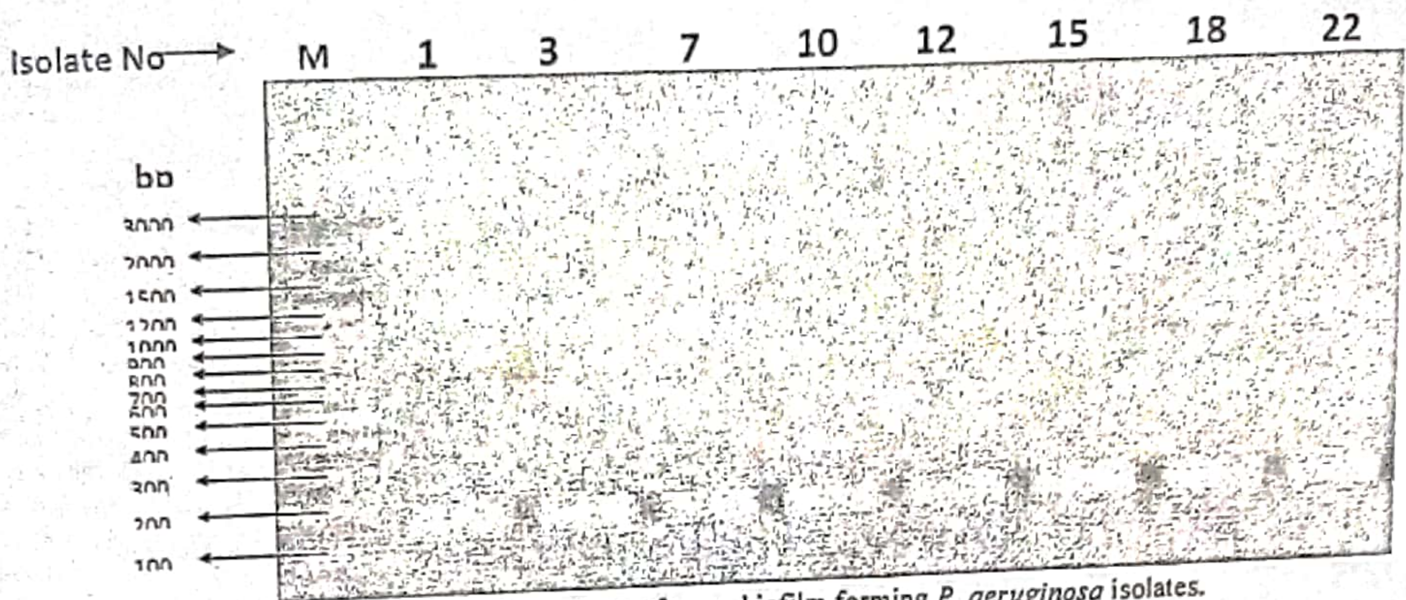

Fig. (6): Detection of *toxA* gene in PCR products of some biofilm forming *P. aeruginosa* isolates.

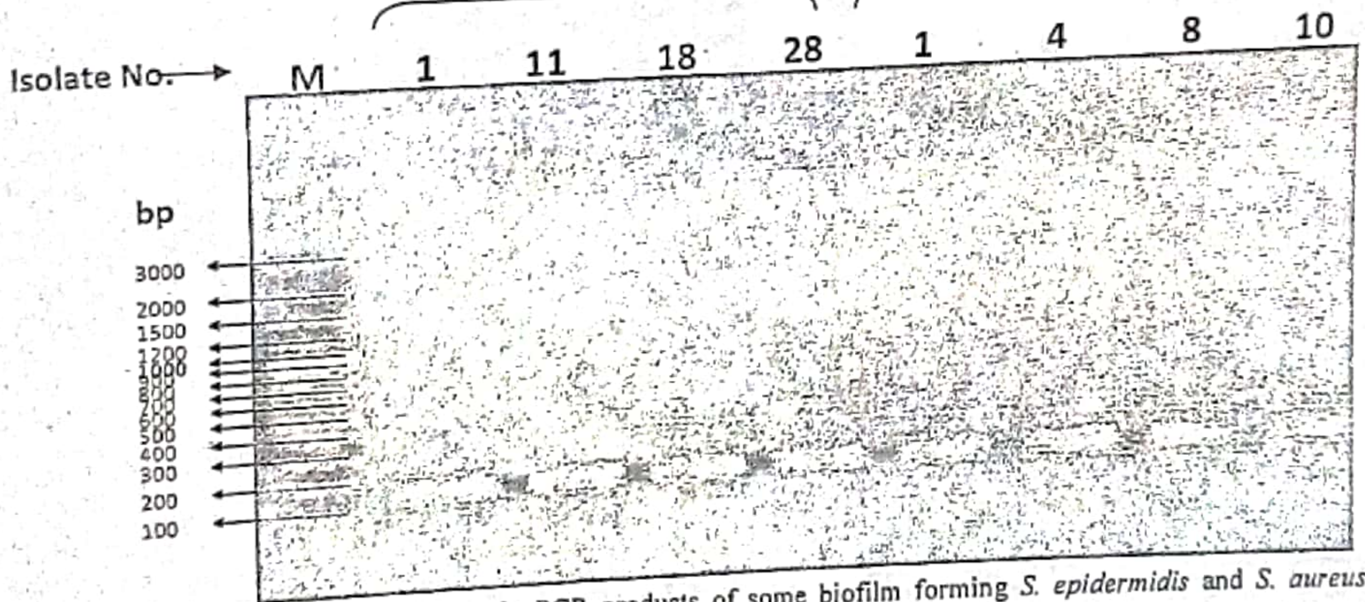

Figure (7): Detection of *icaA* gene in PCR products of some biofilm forming *S. epidermidis* and *S. aureus* isolates.

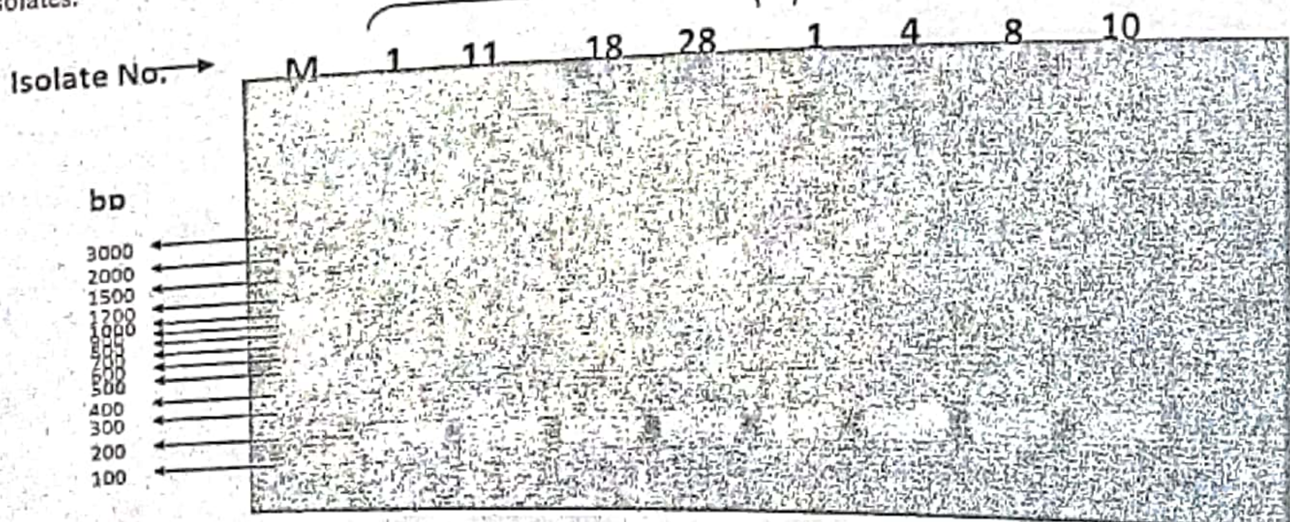

Fig. (8): Detection of *icaA* gene in PCR products of some biofilm forming *S. epidermidis* and *S. aureus* isolates.

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DISCUSSION

Biofilm production is an important pathogenic factor which facilitates the adherence of microorganism to medical devices and protects them from the host immune system and antimicrobial therapy.

The incidence of biofilm production among the total isolates was 119 of 150 isolates at a percent of 80%. It is worthmentioning that different types of microorganisms have the ability to form biofilm and cause CAUTI and show resistance to different types of antibiotics. These results were in consistence with that described previously [10].

In this study, As table 2 revealed, Gram negative bacteria were more predominant than Gram positive bacteria as strong biofilm producers. Where, 36.4% of *Pseudomonas* isolates were highly producers followed by *E. coli* and *Proteus* isolates (30% each). *Klebsiella* isolates were the least highly biofilm forming bacteria among Gram negative isolates. Intresentingly, all Proteus and Pseudomonas isolates were biofilm producers.

However, the strong biofilm producers were less predominant among Gram positive bacteria (table 2). Where 25% of *S. aureus* and 14.3% of *S. epidermidis* isolates were strong producers, respectively.

The detection of different genes associated with biofilm formation on inner surfaces of indwelling catheters by *E. coli*, *K. pneumoniae*, *P. mirabilis*, *Ps. aeruginosa*, *S. epidermidis* and *S. aureus* was performed on the PCR products of these isolates.

E. coli is a major cause of CAUTI and several surface factors might contribute to the biofilm growth by *E. coli* have been characterized. Type 1 fimbriae expression is associated with persistence in the long term catheterized urinary tract [2].lagella are the motility organelles that play a role in the initial adhesion phase of biofilm formation [3]. Moreover, Type 3fimbriae is produced by members of *Enterobacteriaceae* that are associated with opportunistic infection..

Biofilm growth mediated by type 3 fimbriae might be important for the survival of these organisms on the surfaces of urinary catheters. The role of types 3 fimbriae in biofilm formation was confirmed by the complementation of each of these mutants with a plasmid containing the *mrk* genes. Type 3 fimbrial genes *(mrk ABCDF)* was detected at 1000 bp [11] in the PCR products (figure 2).

The importance of type 1 pili as regulatory elements in the ability of *K. pneumoniae* to infect the urinary tract was elucidated [4]. A regulator gene (*fimk*) responsible for biofilm formation and impeding UTI pathogenesis was detected in *K. pneumoniae* isolates.

The *fimk* gene may act exclusively on the expression of type 1 pili or may affect the expression of other adhesins or virulence factors in *K. Pneumoniae* that help in UTI [4].

In this study, the *fimk* gene was detected in the PCR products of biofilm forming isolates of *K. pneumoniae* on agarose gel at band approximately equal to 1000 bp (figure 3).

The role of MR/P fimbriae in *Proteus mirabilis* in colonization of the urinary tract has been established by induction of mutation in a number of these genes [5]. The genes encoding MR/P fimbriae are detected in a complex fimbrial operon [5]. Furthermore, a wild type strain of *Proteus mirabilis* carried two copies of *mrpA* genes has been detected. These data reinforce the importance of MR/P fimbriae in *Proteus mirabilis* UTI.

Analysis of the copies of *mrpA* genes was performed by using the 5mr1 and 3mr1 oligonucleotides primers to amplify DNA flanking the 5 end of *mrpA*. The fragment generated was called mr1 and matched to 991 bp in lenght [12]. In this investigation the detection of mr1 gene in the PCR products of *P. mirabilis* isolates was visualized on agarose gel at band matched to approximately 991 bp (Figure 4).

Similarly, the 5mr2 and 3mr2 oligonucleotides pair was used to amplify the DNA flanking the 3end of *mrpA*. This fragment was called mr2 and equal to 882 bp in length [12]. In this study, mr2 gene was detected at approximzately 882 bp in the PCR products of *P. mirabilis* isolates (Figure 5).

Substantially, *Ps. aeruginosa* isolates recovered from patients suffering from urinary tract infections usually produced significant level of *exotoxin A*. Noticeably, up regulation of *exotoxin A* may be observed within biofilms due to the action of quorum sensing [7,8].

The *toxA* gene of *P. aeurginosa* strains forming biofilm was detected at approximately 200 bp (Figure 6).

All biofilm producing Staphylococcus isolates (*S. epidermidis* and *S. aureus*) were subjected to PCR analysis for detection of *icaA* and *icaD* genes. The detection of *icaA* and *icaD* genes in all biofilm producing strains isolated from catheter segments were observed in PCR products. A band equal to 188 bp and matched to *icaA* was observed (Figure 7). While, a band equal to 198-bp band has been observed on agarose gel (Figure 8) and matched to *icaD* gene [13].

CONCLUSION

Many clinical isolates from urinary catheters were biofilm forming bacteria. Each one of these organisms has a gene responsible for biofilm formation, which increases their ability for causing catheter associated urinary tract infection.

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الكشف عن الجينات المسئولة عن تكوين الغلاف الحيوي (البيوفيلم) في أنواع البكتيريا المختلفة المسببة لتلوث القساطر البولية

أشرف أحمد قدري، منى عبد المنعم السيد، ريهام مجدي جودة

قسم الميكروبيولوجي-كلية الصيدلة-جامعة الزقازيق-الزقازيق-مصر

تهدف الدراسة الحالية إلى الكشف عن البكتيريا المكونة للغلاف الحيوي (البيوفيلم) والناتجة عن تلوث القساطر المنزرعة في الحالب وكذلك الجينات المسؤلة عن تكون هذا الغلاف الحيوي.

لقد تم معرفة وتقييم درجة تكون الغلاف الحيوي (البيوفيلم) في جميع انواع العزلات عن طريق المشاهدة بالعين المجردة لهذا الغلاف وهو مصبوغ على جدار الأنابيب المعملية بصبغة السفرانين وقد أتضح ان 119 عزلة من 150 عزلة تكون الغلاف الحيوي (البيوفيلم).

تمكنت الدراسة ايضا من دراسة الجين المسئول عن تكون الغلاف الحيوي (البيوفيلم) في جميع أنواع العزلات عن طريق استخدام تفاعل البلمرة المتسلسل (PCR) هذا وقد أظهرت نتائج PCR عن وجود الجين المسئول عن تكون الغلاف الحيوي (البيوفيلم) في جميع انواع العزلات وجود جين من ضمن جينات أخرى مسئول عن تكون الغلاف الحيوي (البيوفيلم).

وقد تم تعين *mrkABCDF* جين فى عزلات الايشيريشيا كولاي و*FimK* جين فى عزلات الكليبسيلا. هذا وقد تعيين نسختين من جين *mrpA* في عزلات البروتيس. اظهرت الدراسة أيضا عن الكشف عن جين *toxA* في عزلات السودوموناس المكونة للغشاء الحيوي . بالأضافة الي ذلك لقد تعيين جينات ال *icaA and icaD* في عزلات الأستافيلوكوكس أيوريس والأستافيلوكوكس ابيدرمدز.