

DETECTION OF SOME BIOFILM FORMATION GENES AND THEIR
ROLE IN ANTIMICROBIAL RESISTANCE OF UROPATHOGENIC*Escherichia coli*Reyam H. A.
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ABSTRACT

This study was aimed to evaluate the antimicrobial resistance, biofilm formation and some type 1 fimbriae adhesion genes. A total of 120 urine specimens were obtained from different patients with UTIs during October 2022 to February 2023 from several Baghdad hospitals. Morphological, biochemical and molecular tests were utilized for identifying *E. coli* isolates. The results were shown that only 80 (66.7%) of isolates were identified as *E. coli*. Twelve antimicrobial discs were utilized for evaluate the ability of *E. coli* isolate to resistant these antimicrobials. The results revealed that 98.8% of isolates were resistant to Ampicillin (AMP), followed by 81.3, 77.5, 72.5, 70, 60 and 58.8 of isolates were resistant to Cefepime (FEP), Ceftazidime (CAZ), Tigecycline (TGC), Ciprofloxacin (CIP), Trimethoprim - Sulfamethoxazole (SXT) and Aztreonam (ATM), respectively. All isolates were sensitive to Fosfomycin (FOF) and Amikacin (AMK) as well as the majority of isolates (97.5, 72.5 and 43.8%) were sensitive to Imipenem (IMP), Nitrofurantoin (NIT) and Piperacillin- tazobactam (TZP). A 50 isolates were selected as multi-drug resistant isolates. The biofilm formation of *E. coli* was measured using microtiter plates. The majority of isolates (55%; n=44) were moderate biofilm producers, while 38.75% (n=31) were strong producers and 5 (6.25%) were weak producers. The molecular detections of *fimH* and *fimA* genes were performed with specific primers using PCR technique. The results indicated that all isolates carry both the *fimH* and *fimA* genes.

Keywords: UPEC, *fimH*, *fimA*, UTI, antimicrobials.

عزیز و ابراهیم

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الكشف عن بعض جينات تكوين الأغشية الحيوية ودورها في مقاومة مضادات الميكروبات لبكتيريا الإشريكية القولونية المسببة للأمراض البولية

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باحث

قسم التقنيات الأحيائية، كلية العلوم، جامعة بغداد، بغداد، العراق

المستخلص

هدفت هذه الدراسة الى تقييم مقاومة مضادات الميكروبات و تكوين الأغشية الحيوية و بعض جينات الالتصاق من النوع الأول (fimbriae). تم الحصول على 120 عينة ادرار للعديد من المرضى المصابين بأمراض القناة البولية خلال الفترة من أكتوبر 2022 الى فبراير 2023 من عدة مستشفيات في بغداد. واستخدمت الاختبارات المورفولوجية والبيوكيميائية والجزيئية لتحديد عزلات الإشريكية القولونية. أظهرت النتائج أن ثمانون (66.7%) فقط من العزلات تم تحديدها على أنها *E. coli*. تم استخدام اثني عشر قرصاً مضاداً للميكروبات لتقييم قدرة عزل الإشريكية القولونية على مقاومة مضادات الميكروبات هذه. كشفت النتائج ان 98.8% من العزلات كانت مقاومة لأمبيسيلين، تليها 81.3 و 77.5 و 72.5 و 70 و 60 و 58.8% من العزلات مقاومة للسيفتازايم وسيفتازايميتيم والتيجيسايكلين والسايبروفلاكسين وترايمثوبرين-سولفاميثوكسازول وازترونام على التوالي. كانت العزلات جميعها حساسة للفوسفومايسين والاميكاسين بالإضافة الى ان اغلب العزلات (97.5 و 72.5 و 43.8%) كانت حساسة للاميبينيم والنايتروفورانتونين والبايراسايلين-تازوباكتيم. تم اختيار 50 عزلة كعزلات مقاومة للأدوية المتعددة. تم قياس إمكانات تكوين الأغشية الحيوية للقولونية باستخدام ألواح المعايرة الدقيقة. وكانت غالبية المعزولات (55%؛ عدد = 44) من منتجي الأغشية الحيوية المعتدلة، في حين أن 38.75% (عدد = 31) كانوا منتجين أقوياء و 6.25% كانوا منتجين ضعفاء. تم إجراء الكشف الجزيئي عن جينات *fimH* و *fimA* باستخدام بادئات محددة باستخدام تقنية تفاعل البوليميراز المتسلسل. أشارت النتائج إلى أن جميع العزلات تحمل كلا من جينات *fimH* و *fimA*.

كلمات مفتاحية: UPEC, *fimH*, *fimA*, إصابة القناة البولية، مضادات ميكروبية.



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INTRODUCTION

Escherichia coli is one of the most researched microbes (2, 8). Uropathogenic *Escherichia coli* (UPEC) is a common cause of urinary tract infections (12). These strains demonstrate a wide variety of virulence factors and tactics. These *E. coli* strains are commonly known as UPEC because of their uropathogenicity. The most prevalent urological and renal condition is urinary tract infection (UTI) (7, 11). It is frequently linked to morbidity in both inpatient and outpatient settings. UPEC is the most frequent bacteria in between 50 and 90 percent of all simple UTIs (12). The urinary tract infection caused by UPEC is facilitated by a variety of virulence factors, such as fimbrial and non-fimbrial adhesins, toxins, iron acquisition factors, LPS, and capsules (16). Important virulence factors in *Escherichia coli* strains are the type 1 fimbriae responsible for bacterial pathogenicity and biofilm development. Over ninety-five percent of all *E. coli* strains express type 1 fimbriae (1, 17). The fibre of a type 1 pilus is formed by four distinct protein subunits (FimH, FimG, FimF, and FimA), all of which remain in noncovalent connection with one another. FimH, an adhesin, is located at the fiber's tip, along with the linker subunits FimG and FimF, which together make up the pilus's short, flexible fibrillar tip that is linked to the rod, FimA (34, 36). Biofilm-related infections are associated with increased mortality and morbidity due to the spread of bacteria from infected in-hospital medical devices (6, 30). Bacterial communities known as biofilms are encased in a self-made extracellular matrix consisting of micro-molecules (such as DNA), proteins and exopolysaccharides (EPSs). They are capable of growing on both living and nonliving substrates (15, 24). Antimicrobial resistance among bacteria strains is a growing problem all over the world. Acquired resistance to three or more classes of antimicrobials, or multidrug resistance (MDR), is a major barrier to

effective patient care today (32, 35). With the advent of *E. coli* came a surge in resistance to multiple antimicrobials making antimicrobial resistance in UPEC a global health crisis. An alarming level of antimicrobial resistance is developing in UTI pathogens due to the widespread and indiscriminate use of antimicrobials, as well as the practice of prescribing antimicrobials to treat UTI without bacterial characterization. Therefore, familiarity with epidemiological data regarding the sensitivity of uropathogens to antibacterial drugs is crucial for the prompt beginning of suitable empirical treatment (3, 5). This study was aimed to detect some genes of biofilm formation and reveal their roles in antimicrobial resistance of UPEC in patients with UTI.

MATERIALS AND METHODS

Collection of clinical specimens

A 120 clinical specimens of urine were obtained from Iraqi individuals with UTIs, during the duration between (October 2022 – February 2023) from hospitals of Medicine City in Baghdad. All specimens were maintained in sterile containers and transferred into laboratory.

Diagnosis of clinical specimens

All clinical specimens were subjected into several diagnostic tests, including gram stain, features of bacterial colonies on media, including MacConkey agar (Neogen/USA), CHROM agar (Himedia/India) and Eosin Methylene Blue (Himedia/India), as well as some biochemical tests (Himedia/India), including Simmons citrate test, urease, catalase, oxidase and Indole tests.

Antimicrobial susceptibility test

This test was carried out using the disc diffusion technique, as outlined in recommendations depend on the Clinical and Laboratory Standards Institute (20). Types and concentrations of antimicrobial discs, which supplied by Himedia/India, were utilized in this work as listed in Table (1):

Table 1. The antimicrobials that utilized in this study.

No.	Antimicrobial Disc	Symbol	Disc content (µg/disc)
1	Ampicillin	AMP	10
2	Trimethoprim - Sulfamethoxazole	COT (SXT)	1.25/23.75
3		CAZ	30
4		IPM	10
5	Aztreonam	ATM	30
6	Cefepime	FEP	30
7	Fosfomycin	FOF	200
8	Ciprofloxacin	CIP	5
9	Nitrofurantoin	NIT	300
10	Amikacin	AK (AMK)	30
11	Piperacillin- tazobactam	PIT	100
12	Tigecycline	TGC	15

Assessment of biofilm formation

Quantitative determination of biofilm formation was determined by a colorimetric microtiter plate assay. This method was carried out in accordance with (14).

1. All bacterial strains were cultured for 24 hours at 37°C in brain heart infusion broth. For adjusting the turbidity to McFarland standards (0.5), 100µl of bacterial growth was transferred to a 2 ml tube of normal saline.

2. Sterile 96-well polystyrene microtiter plates with flat bottoms were filled with 180µl of tryptic soy broth containing 1% glucose.

3. Three wells of a sterile 96-well polystyrene microtiter plate with a flat bottom were inoculated with 20µl of bacterial suspension (prepared in normal saline). Six negative control wells (bacteria-free) filled with brain heart infusion broth.

4. Without disturbing the plates throughout their 24-hour aerobic incubation at 37°C, they

were sealed with their lids. After incubation, all plates were washed with phosphate buffer saline for three times before being dried.

5. To fix the biofilms, 200 µl of methanol was added to each well, and after 15 minutes at room temperature, the methanol was poured out and the plate was permitted to dry.

6. The plates were stained for 15 minutes at room temperature with 200 microliters of a crystal violet solution containing 0.1 %. In addition, the plate was let to dry after washing the wells to get rid of any remaining colour.

7. For 10 minutes, 200µl of 33% acetic acid was used to dissolve the colour.

8. The optical density (OD) of each well was measured at 630nm using an ELISA reader.

9. Table (2) displays the results of an ELISA reader set at 630 nm, which were interpreted as strong, moderate, or weak biofilm producers.

Table 2. Interpretation Adherence of biofilm formation based upon the OD values

Adherence of Biofilm Formation	Interpretation
$OD_s \leq OD_c$	Non –adherent
$OD_c < OD_s \leq 2 * OD_c$	Weakly adherent
$2 * OD_c < OD_s \leq 4 * OD_c$	Moderately adherent
$4 * OD_c < OD_s$	Strongly adherent

* OD = optical density; OD_c = optical density cut off.

* Mean OD of negative control + 3× SD of the negative control.

Extraction of DNA: EasyPure^(R) Bacteria Genomic DNA kit (TRANS/China) was utilized for extraction of DNA from specimens and the procedure was performed according to recommendations of manufacturers.

Molecular detection of 16S RNA, *fimH* and *fimA* genes: Adhesion genes (*fimH* and *fimA*) in *E. coli* isolates were identified using the

PCR method. These genes were amplified at 80 V for 80 minutes while being monitored by 2% agarose gel electrophoresis. Primers were supplied by Macrogen® (Korea). Table (3) shows the primer sequence and amplicon sizes, and Table (4) details the PCR program's execution.

Table 3. Primer's sequence of (*fimH* and *fimA*) gene and amplicon sizes

Name of Primer	Sequence (5' – 3')	Product Size (bp)	Reference
16S rRNA	F: TTATCCCCCTCCATCAGGCAG R: ATGGCTCAGATTGAACGCTGG	134	This study
<i>fimA</i>	F: CTGGCAATCGTTGTTCTGTCG R: TCAACAGAGCCTGCATCAACT	140	This study
<i>fimH</i>	F: ATTGCCGTGCTTATTTTGC GA R: ATTGGCACTGAACCAGGGTAG	167	This study

Table 4. The PCR analysis program for the primers.

The steps	Temperatures (°C)	Period	No. of cycles
Initial Denaturation	94.0	5 minutes	
Denaturation	94.0	30 second	
Annealing	53 ¹ , 54 ² , 56 ³	45 second	30
Extensions	72.0	45 second	
Final extensions	72.0	7 minutes	

Optimal annealing temperature for ¹*fimH*, ²*fimA*, ³16S rRNA

The reaction of PCR was carried out in a gradient thermocycler (Eppendorf -Germany). This step was carried out by adding 12.5 µl from OneTaq®2X Master Mix (NEB®/England), 3 µl of DNA sample, 1 µl 10 pmol/µl from each primer and 7.5 µl of free-nuclease water were utilized into reaching the total volume of 25µl.

RESULTS AND DISCUSSION

Collection, isolation and identification of clinical Specimens: A 120 urine specimens were obtained from patients. Only 66.7%

(n=80) isolates were identified as *E. coli* according to results of diagnosis. Based on the results, these bacterial isolates were gram-negative rod and appeared as pink colonies on both MacConkey agar and CHROM agar as well as green metallic sheen colonies on EMB, as shown in Figure (1). In addition, these isolates were given positive for indole and catalase tests and negative for oxidase, urease and Simmons citrate tests. VITEK2 system was utilized to ensure that these isolates were *E. coli*.

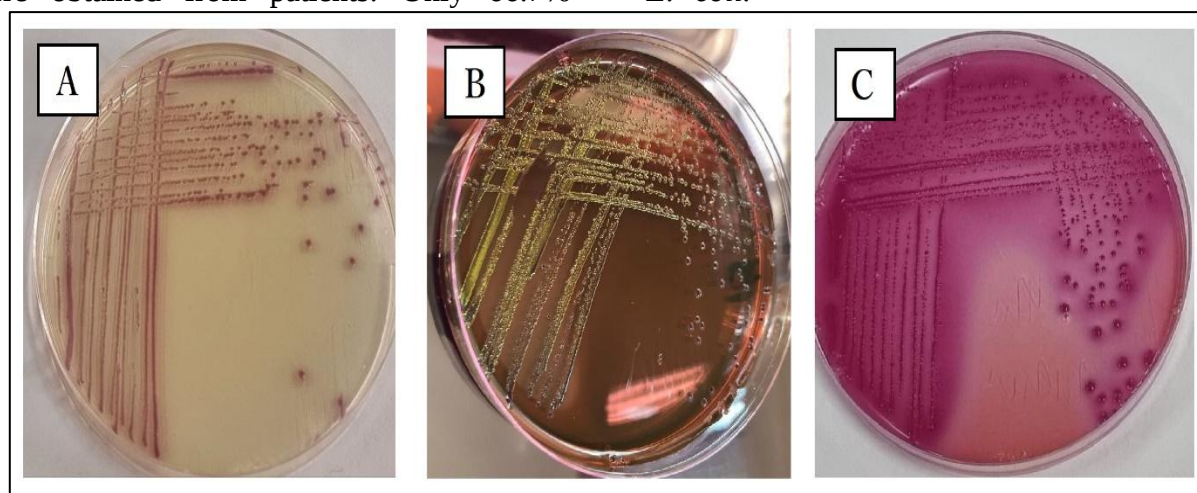


Figure 1. The colonies of *E. coli* on different media (A: CHROM agar, B: EMB, C: MacConkey gar).

Results of antimicrobial susceptibility test

Different discs of antimicrobials were used in order to investigate the antimicrobial resistance of *E. coli* isolates. The results were showed that there were several degrees of resistance of *E. coli* isolates against common utilized antimicrobials. Based on the results in Figure (2). 98.8% of isolates were resistant to AMP, followed by 81.3, 77.5, 72.5, 70, 60 and 58.8 of isolates were resistant to FEP, CAZ,

TGC, CIP, SXT and ATM, respectively. All isolates were sensitive to FOF and AMK as well as the majority of isolates (97.5, 72.5 and 43.8%) were sensitive to IMP, NIT and TZP. The prevalence of drug-resistant *E. coli* UTIs in both community and inpatient settings is cause for serious issues (10). According to these results, all isolates were sensitive to AMK, which is agreed with (21). The resistance of isolates against SXT was 70%

which is included in range of 40 to 80% as a study reported by (4). Furthermore, it has been reported that 50.5% of isolates were resistant to SXT (9), whereas this result agreed with the result of the current study. Multi-

resistant strains are more likely to propagate when antimicrobial resistance genes are shared across bacteria via plasmids, transposons, and integrons (25).

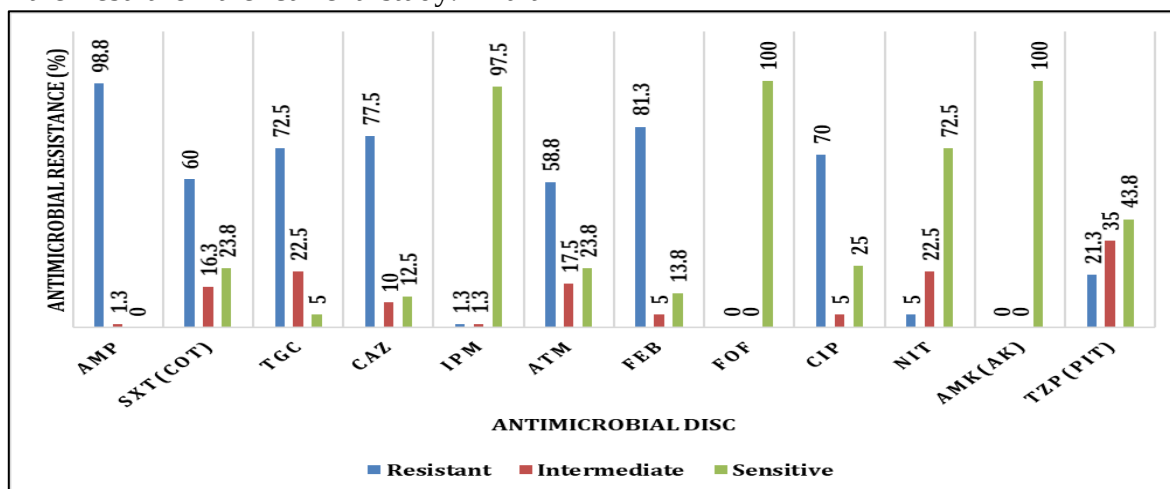


Figure 2. Pattern of antimicrobial resistance in *E. coli* isolates, Trimethoprim (AMP), Trimethoprim/Sulfamethoxazole (COT or SXT), Ceftazidime (CAZ), Imipenem (IMP), Aztreonam (ATM), Cefepime (FEP), Fosfomycin (FOF), Ciprofloxacin (CIP), Nitrofurantoin (NIT), Amikacin (AK or AMK), Piperacillin-tazobactam (PIT or TZP).

In this study, only 50 isolates were selected as multi-drug resistant isolates, whereas these isolates were utilized for further experiments.

DNA extraction

The method of DNA extraction with EasyPure® Genomic DNA Kit was carried out according to the protocol provided by the manufactured company (Transgene®/China), then the concentration was measured using Qubit 0.4 (Invitrogen/USA). The results revealed that the DNA concentration of all isolates were ranged between 14.2-22 ng/μl.

Molecular detection of 16S rRNA gene

The 16S rRNA gene was detected molecularly using the PCR technique, in order to confirm the identification of *E. coli*. As shown in Figure (3). All isolates were harbored 16SrRNA and identified as *E. coli*. On the agarose gel images of all isolates, a single 134-bp band was observed for indicate the presence of the 16S rRNA gene.

It has been reported that 90% of isolates were identified as *E. coli* using 16S rRNA gene detection, considering that this technique is a good tool for identifying these bacteria (23).

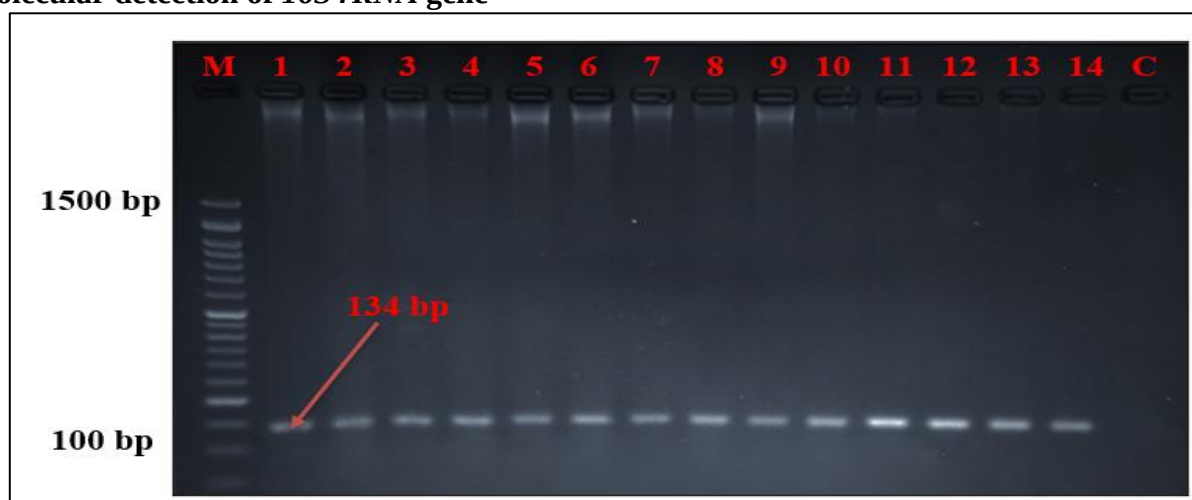


Figure 3. Gel electrophoresis in 2% agarose (80 min, 80 V) for 134 bp of 16S rRNA amplification using PCR, M: DNA ladder, Line (C): Negative control, Lines (1-14): isolates were positive for 16S rRNA.

Formation of Biofilm

The biofilm formation of *E. coli* was measured using microtiter plates. The majority of isolates (55%; n=44) were moderate biofilm

producers, while 38.75% (n=31) were strong producers and 5 (6.25%) were weak producers (Table 5).

Table 5. The distribution of biofilm formation among isolates

Biofilm producer	N=80	Percentage (100%)
Non	0	0
Weak	5	6.25
Moderate	44	55
Strong	31	38.75

Indirectly, biofilms contribute to bacterial antimicrobial resistance by improving the pharmacokinetic qualities of the anatomical region (i.e. the capacity of these medications to penetrate and reach the microorganisms in effective quantities) (19, 37). It is not surprising that genes involved in biofilm-development are QS-mediated, given that biofilm formation is a cooperative behaviour that benefits the bacteria as a whole and increases their chances of survival (26, 27). There are fundamental distinctions between biofilm and planktonic cell growth, such as compositional variances in biomass, that make direct comparisons between the two modes of growth challenging. During *in vitro* testing, biofilm-producing and non-producing bacteria of the same species may show distinct resistance patterns due to these evolutionary trade-offs (27). The ability to produce extracellular polysaccharide (EPS) matrix is

highly correlated with the tendency of these bacteria to become nosocomial pathogens (*Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Acinetobacter* spp., *Staphylococcus aureus*, and others), allowing them to survive in the harsh physical settings of hospitals (13, 33). Differential expression of virulence proteins, metabolic end-products, or antimicrobial-resistance determinants may result from transcriptional alterations in these bacteria (typically mediated by quorum-sensing [QS]-based mechanisms) that occur during biofilm development (26, 38).

Distribution of *fimH* and *fimA* genes among *E. coli* isolates :Specific primers were used in a polymerase chain reaction (PCR) assay for molecular detection of the *fimH* and *fimA* genes. All of the isolates of *E. coli* were subjected to this test, and as shown in Figure (4) and Figure (5), the results indicated that all isolates carry both the *fimH* and *fimA* genes.

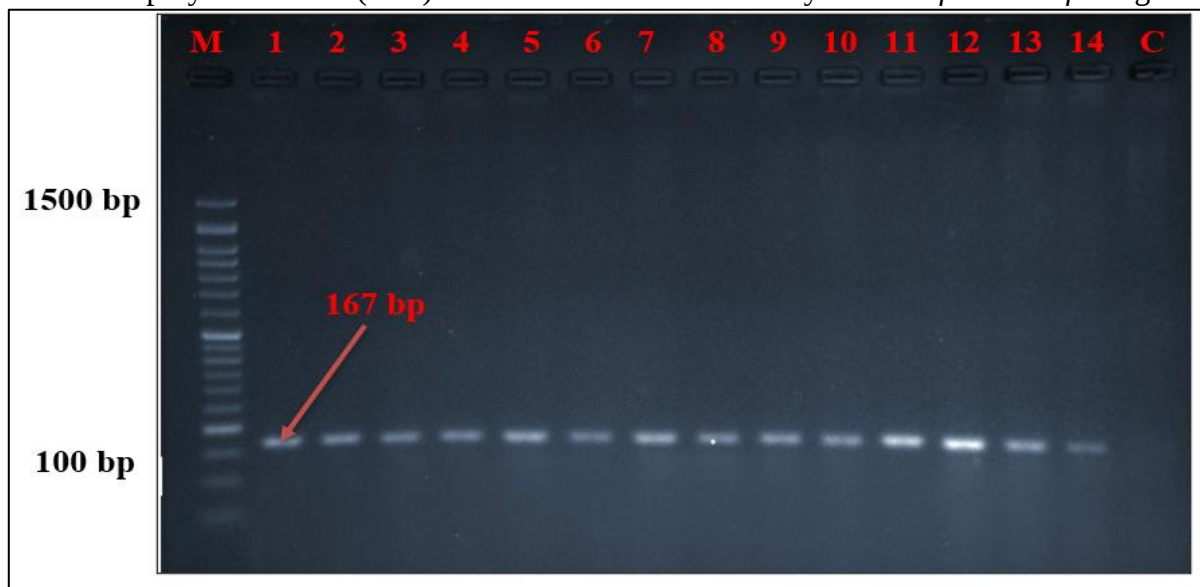


Figure 4. Gel electrophoresis in 2% agarose (80 min, 80 V) for 167 bp of *fimH* amplification using PCR, M: DNA ladder, Line (C): Negative control, Lines (1-14): isolates were positive for *fimH*.

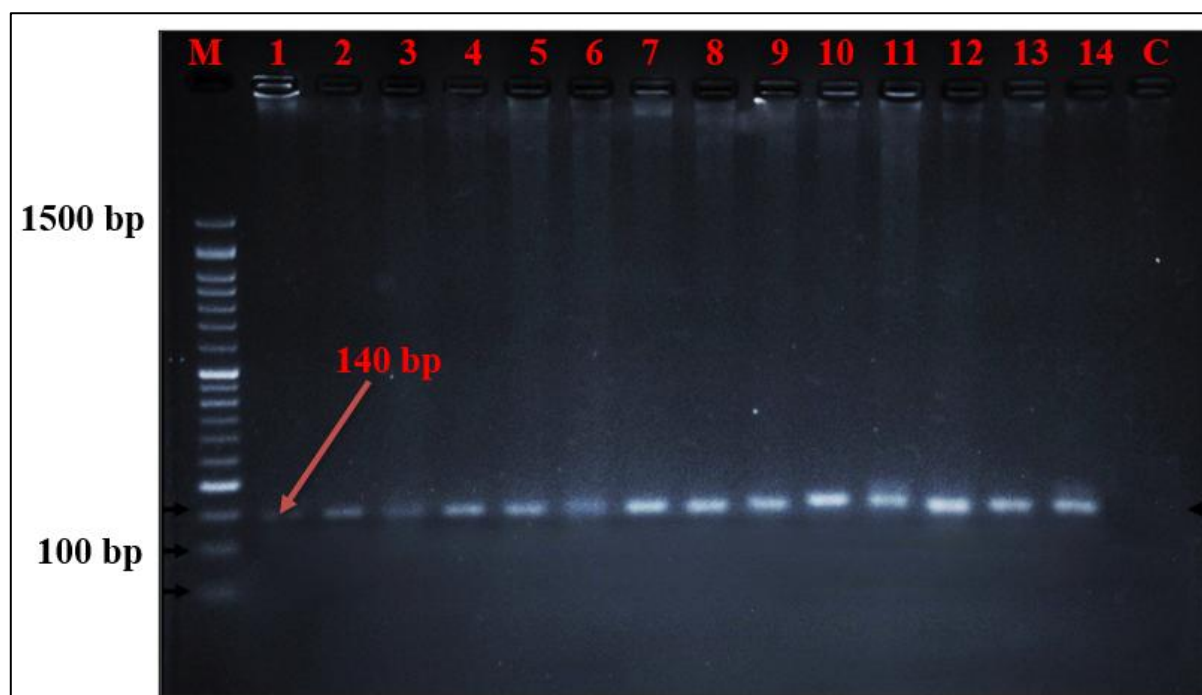


Figure 5. Gel electrophoresis in 2% agarose (80 min, 80 V) for 140 bp of *fimA* amplification using PCR, Line (M): DNA ladder, Line (C): Negative control, Lines (1-14): isolates were positive for *fimA*.

All MDR isolates, which harbored both *fimA* and *fimH* genes, were strong biofilm producers (62%; n=31) and moderate biofilm producers (38%; n=19). Many adherence molecules, including pili, fimbriae, lipopolysaccharides, and capsular polysaccharides, help uropathogenic *E. coli* (UPEC) manage adherence in various environments and organise biofilms, making it a major cause of catheter-associated urinary tract infections (CAUTIs). The initial phase in a bladder infection caused by UPEC is adhesion to the surface host urothelial via type 1 fimbriae (22, 40). It was found that *fimH* was present in all isolates tested, whereas *fimA* was present in 31.6% of specimens (18). Also, only 44% and 86.3% of isolates were carry *fimA* gene as reported by (31) and (28), respectively. It is generally agreed that the adhesions of *E. coli* are the primary contributors in its pathogenicity. The ability of these compounds to trigger bacterial-host cell communication pathways aids bacterial invasion and tissue colonisation (29, 39).

CONCLUSIONS

In this study, *E. coli* isolates recovered from urine samples of Iraqi patients with urinary tract infections showed a high prevalence of the *fimH* and *fimA* genes, indicating their widespread presence as key virulence factors.

The detection of these adhesin genes was associated with variations in antimicrobial susceptibility, suggesting a potential link between genetic determinants of adhesion and resistance patterns. The high frequency of these genes among clinical isolates highlights their possible role in enhancing bacterial survival under antimicrobial pressure, thereby contributing to treatment challenges. These findings underscore the need for continuous monitoring of virulence genes alongside resistance profiling to improve therapeutic strategies and limit the spread of multidrug-resistant *E. coli* in the community and healthcare settings.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

DECLARATION OF FUND

The authors declare that they have not received a fund.

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