Journal of Applied and Natural Science
14(4), 1485 - 1492 (2022)
ISSN : 0974-9411 (Print), 2231-5209 (Online)
journals.ansfoundation.org

Research Article

Molecular detection of extended spectrum ß-lactamase genes in *Escherichia coli* isolates from urinary tract infected patients

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Article Info
https://doi.org/10.31018/jans.v14i4.3948
Received: August 17, 2022
Revised: December 4, 2022
Accepted: December 9, 2022

How to Cite
Hussein, F. A. N. and Alwash, M. S. (2022). Molecular detection of extended spectrum ß-lactamase genes in *Escherichia coli* isolates from urinary tract infected patients. *Journal of Applied and Natural Science*, 14(4), 1485 - 1492. https://doi.org/10.31018/jans.v14i4.3948

Abstract
Extended-spectrum ß-lactamases (ESBL) are a major source of concern. ESBL have been recorded around the world. Globally, the number of people infected with Enterobacteriaceae that produce extended-spectrum beta-lactamase (ESBL) is on the rise. It has been a rise in resistance to ß-lactam antibiotics among them. In this study, the objective was to collect *Escherichia coli* isolates from Urinary tract infection patients using selective medium, determine the prevalence of ESBL-producing *E. coli*, phylogenetic groupings of isolates, ESBL production, and biofilm formation among the isolates of *E. coli* isolates. The study included 250 *E. coli* samples from male and female subjects and grown on a selective medium. The isolated bacteria were submitted to different tests, including the detection of biofilm development and testing of the phylogenetic grouping of the *E. coli* isolate using triplex-PCR analysis. Representatives of each isolate were phenotypically evaluated for antibiotic resistance and classified into phylogenetic groupings. The results of extended-spectrum ß -lactams antibiotics showed the greatest resistance levels. There were 100% resistance rates for Ceftazidime-Clavulantae (CZC) and Cefotaxime-Clavulantae (CTC), 78.7% for Ceftazidime (CAZ), 86.7% for Cefotaxime CTX, 84% for Aztreonam (ATM), 87.3% for Ceftriaxone (CRO) and 83.3% for Cefpodoxime (CPD). *E. coli* isolates belonging to phylogroup B2 (91, 91%), and subtyping B23 (75, 75%) were the most common among UTI patients. ESBL-producing *E. coli* isolates were prevalent in individuals with UTIs. Most *E. coli* isolates from UTI patients at Al-Hillah hospitals belonged to phylogroup B2, followed by D, B1, and A. B2 was the most prevalent group in the study. This study examined the dissemination of ESBL genes in phylogenetic groups of the *E. coli* isolates from UTIs patients in the Al-Hillah, Iraq.

Keywords: Biofilm formation, *Escherichia coli,* Extended spectrum ß -lactams Phylogenetic groups, Uropathogenic

INTRODUCTION

Beta-lactams)β-lactams) are among the antibiotics that are recommended to Uropathogenic *Escherichia coli* (UPEC) patients in Iraq and around the world the most frequently. The group may be identified by the presence of a beta-lactam ring in the structure of their molecules. Penicillins, cephalosporins, carbapenems, monobactams, and beta-lactamase inhibitors are antibiotics. These antibiotics can target Gram-positive and Gram-negative bacteria (e.g., many Enterobacteriaceae) (Pandey and Cascella, 2021). Extended-spectrum lactamases (ESBL) produced by Enterobacteriaceae compromise the capacity to treat an infection worldwide (Teklu *et al*., 2019). Antibiotics such as penicillin, broad -spectrum cephalosporins and monobactams can be hydrolyzed by ESBL-producing bacteria; however clavulanic acid, a β-lactam inhibitor, can block their activity (Paterson and Bonomo, 2005; Bush and Fisher, 2011). In addition, ESBLs are a huge group of plasmid-mediated, varied, complicated, and quickly changing enzymes that provide an enormous therapeutic challenge in treating both hospital-acquired infections and those acquired in the community (Chandel *et al*., 2011). ESBL producers can cause anything from a simple urinary tract infection to sepsis, which can be fatal (Teklu *et al*., 2019). Infections produced by ESBL-producing bacteria pose a rising threat to the spread of resistant strains in humans and animals, raising concerns for global public health (Abayneh *et al*., 2018). The problem is severe in developing countries such as Iraq, where the drugs can be obtained without prescription due to a lack of drug regulation (Alwash and Al-Rafyai, 2019). In comparison with the rest of the world,

there is generally a lack of comprehensive data regarding ESBL-producing Enterobacteriaceae in Iraq. There is currently no standard screening for ESBLs in Al-Hillah, Iraq, clinical labs. ESBL-producing bacteria must be routinely identified and monitored in a clinical laboratory. In addition, Abbas 2016 and Abbas 2019 are the two studies that have reported on ESBL-producing Enterobacteriaceae from lower respiratory tract infection patients and hospital teaching hospital environments respectively in AL-Hillah city, Iraq. Therefore, this study was carried out to establish the prevalence and antibiotic use in urinary tract infection (UTI) as well as to determine the resistance pattern of ESBL-producing *Escherichia coli. coli* isolates from UTI's patients in Al-Hillah city, Iraq

MATERIALS AND METHODS

Sample collection

From November 2021 to March 2022, two hundred fifty (250) urine specimens were collected from patients for testing purposes. The patients who have UTI, visited private laboratories and hospitals in the Babylon Province. Patients with symptomatic UTIs were asked to provide 10 ml of midstream urine specimens, which were then collected in disposable sterile tubes for testing. All of the samples were tested within 30 minutes of being taken, or they were put in the fridge at 4°C until further analysis. Each sample was grown directly on a variety of media, such as MacConkey agar (MA; Himedia, India). The MacConkey was used to measure the ability of bacterial strains to ferment lactose and make pink colonies (MacFaddin,2000) .The Eosin methylene blue agar (EMB; Himedia, India) was used to differentiate *E. coli* from other organisms by producing green metallic sheen colonies. Plates were incubated for 24 hours at 37°C.

Diagnosis of *E. coli* by GN-ID with VITEK-2 compact

The Vitek 2 Compact System from Biomerieux in France was used to confirm the biochemical tests. The assay was performed according to the instructions from the manufacturer. Both instruments can automatically identify bacterial isolates. This kit was used to discover bacteria, and the steps were to put 3 ml of normal saline in a test tube and put a loop full of an isolated colony in the tube. Colonies need to be 24 hours old. The test tube was put into a dens check machine so that the colony could be standardized to McFarland's standard solution (1.5×10^8 CFU/ml).The standard inoculums were put in the cassette (20 well microplates), and a barcode was used to enter a sample's identification number into the computer software. VITEK 2 card had the sample ID number linked to it, and when the cards were filled, the cassette was moved from the filler module to the reader incubator module (Moehario *et al*., 2021).

Antimicrobial susceptibility testing and screening for ESBL

Antibiotic susceptibility testing was carried out in accordance with the instructions provided by the Clinical and Laboratory Standards Institute (CLSI, 2014). The *in vitro* susceptibility of 150 *E. coli* isolates to 7 antimicrobial agents: Aztreonam (ATM, 30µg), Ceftriaxone (CRO, 30µg), Ceftazidime (CAZ, 30µg), Cefotaxime (CTX, 30µg), Cefpodoxime (CPD, 10µg) Ceftazidime/ Clavulantae (CZC, 30/10µg), Cefotaxime/Clavulantae (CTC, 30/10µg) were tested according to Clinical and Laboratory Standards Institute instructions (CLSI, 2021). The bacterial isolates were grown on MacCkonkey agar and then subcultured in brain heart infusion broth for 24 hours at 37°C. The turbidity was adjusted in 0.85% sterile normal saline solution to 0.5 McFarland's standard (10^8 CFU/mL) and then cultivated using a cotton swab on Mueller Hinton agar MHA (Liofilchem, Italy). The antibacterial activity was measured as the mean of the inhibition zone diameter in millimetres after the antibiotic discs were applied to MHA using a sterile forceps and pushed down to achieve complete contact with the agar (mm).The plates were incubated for 24 h at 37°C. The next day, the zone diameter was measured and classed as either sensitive or resistant by comparison to CLSI.

Biofilm formation assay

This technique was used to measure the biofilm production of all *E. coli* isolates. The medium contained brain heart infusion agar, sucrose and Congo red dye. The examined organisms were grown on Congo Red Agar ((Himedia, India) and incubated at 37°C for 24 to 48 hours. A positive result was indicated by black colonies with a dry crystalline consistency, while non-biofilm producers usually remained pink crystalline colonies (Jain and Agarwal, 2009).

Extraction of genomic DNA

Extraction of DNA from *E. coli* isolates was carried out using the Favor Prep™ Genomic DNA Mini Kit (Favorgen, Taiwan). After 24 hours of incubation, *E. coli's* DNA was extracted. The DNA quality and quantity were evaluated by utilising a NanoDrop (Memmert, Germany). When the genomic DNA was eluted, it was kept at -20°C until further processing.

Molecular identification of phylogentic group of *E. coli* isolates

According to Clermont *et al.* (2000), all *E. coli* isolates were categorized to either one of four major phylogenetic groups (A, B1, B2, or D) through triplex PCR us-

ing a template DNA forward and reverse primers, nuclease free water, master mix and $Mgcl_2$. Mixing all PCR components in one tube was done in accordance with the manufacturer's recommendations. Triplex PCR was evaluated the genetic markers presence *chu*A and *yja*A and the TspE4.C2 DNA fragment .The reaction mixture (20 µL) contained Master Mix (8 µL), $Mgcl_2$ (7.5 µL) , Nuclease-free water (0.5 µL), Template DNA (2 µL), Primer Forword (10 picomols/ 1 µL), Primer Reverse (10 picomols/ 1µL). The program of PCR was as follows 4 minutes at 94 °C and 1 cycle for Initial denaturation, followed by 30 cycles for 20 second at 94°C for DNA denaturation and 30 seconds at 59°C for Primer annealing, and 1 minute at 72°C for Extension step followed by Final extension at 5 minutes at 72°C for 1 cycle. The amplified products were separated in a 1.5% agarose gel using ethidium bromide. The gel was photographed under UV light after electrophoresis. *E. coli* isolates were classified into A (TspE4.C2-, *chu*A-) B1 (TspE4.C2+, *chu*A-,) B2 (*yja*A+, *chu*A+,) and D (*yja*A-, *chu*A+) groups. In order to boost the differentiation between individual isolates, the following was done in order to create subgroups: subgroup A0 (group A), TspE4.C2−, *yja*A−,chuA−, subgroup A1 (group A), TspE4.C2−, *yja*A+, *chu*A− , group (B1), TspE4.C2+, *yja*A−, chuA−; subgroup B22 (group B2), TspE4.C2−, *yja*A+, chuA+, subgroup B23 (group B2), TspE4.C2+, *yja*A+, *chu*A+; subgroup D1 (group D) TspE4.C2−, *yja*A−, chuA+, and subgroup D2 (group D) TspE4.C2+, *yja*A−, *chu*A+ (Alwash and Al-Rafyai, 2019).

RESULTS AND DISCUSSION

Isolation and Identification of *Escherichia coli* Isolates

The results of present study (Fig. 1) showed that *E. coli* on MA produced pink colonies due to lactose fermentation lead to the production of acidic metabolic products, which lower the pH and turn the colonies pink as reported by Murray *et al*. 2020). *E. coli* isolates when cultured on EMB agar produced a green metallic sheen colonies, because the dyes make a dark purple complex when they are in an acidic environment, which is usually linked to a green metallic sheen faecal coliforms are known for their capacity to ferment lactose and/or sucrose, and their metallic green sheen is a sign of this (Lal and Cheeptham, 2007). The present results indicated that *E. coli* was the most prevalent cause of UTIs (60 %). Sharma *et al* (2016) in India noted that *E. coli* accounted for 68% of all clinically relevant urine isolates, followed by *Klebsiella pneumoniae* (14%), *Proteus sp.* (6%), and *Pseudomonas aeruginosa* (3%). Several studies (Foxman, 2014; Klein & Hultgren, 2020) noted that *E. coli* was found in 80% of midstream urine samples from people in different countries with UTIs. ExPEC's dominance over other gram-negative bacteria can be explained by the development of virulence factors that increase their ability to colonise the urinary system and causes illness. UPEC isolates have a variety structural such as (flagella, bili , curli) and produced (toxins, enzymes, iron acquisition system) virulence characteristics the ability to attach to host cells in urinary tract constitutes the first most crucial factor of pathogenicity (Terlizzi *et al*., 2017).

Antibiotic susceptibility profile

Detection for ESBL-producing *E. coli* (150) isolates initially screened for ESBL production using the phenotypic method, and then their ESBL production validation using the phenotypic confirmatory test (CLSI, 2014), showed that (109, 72%) had resistance for ESBL-producing, whereas (41, 28%) were sensitive for ESBL-producing . Disc diffusion against 109 isolates displayed ESBL-producing resistance The results revealed that the resistance was 100% for Ceftazidime-Clavulantae(CZC) and Cefotaxime-Clavulantae (CTC), 78.7% for Ceftazidime (CAZ), 86.7% for Cefotaxime (CTX), 84% for Aztreonam (ATM), 87.3% for Ceftriaxone (CRO) and 83.3% for Cefpodoxime (CPD) (Fig. 2). The antibiotic susceptibility results in Table 1 demonstrated that the majority of *E. coli* isolates were resistant to a number of different antibiotics. Increased use of antimicrobials in health settings, notably third-generation cephalosporin, has been linked to an increase in ESBL-producing Enterobacteriaceae has

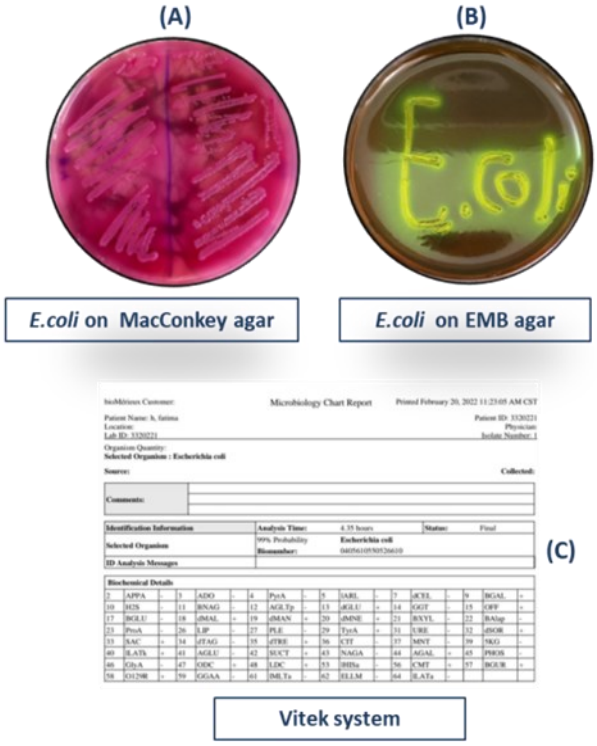

Fig. 1. *Isolation and identification of E. coli isolates using (A) MacConkey agar, (B) EMB agar, and (C) Vitek system*

Table 1. Resistance profiles of phenotypic ESBL-producing *Escherichia coli* isolates

Antibiotics	No. of resistant isolates	Resistance %
Aztreonam (ATM)	126	84%
Ceftriaxone (CRO)	131	87.3%
Cefpodoxime (CPD)	125	83.3%
Ceftazidime (CAZ)	118	78.7%
Ceftazidime-Clavulantae(CZC)	150	100%
Cefotaxime (CTX)	130	86.7%
Cefotaxime-Clavulantae(CTC)	150	100%

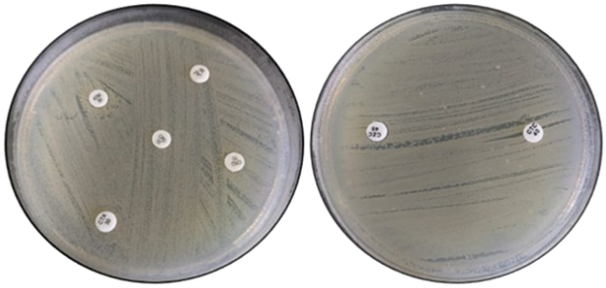

Fig. 2. *ESBL-producing in E. coli Isolates*

Fig. 3. *Congo red agar plates showing biofilm producing E. coli isolates*

been reported in the world. Bacterial resistance to ß-lactam antibiotics looked to be universal, according to a number of research results (Ejaz *et al*., 2021). Kumar (2021) showed that ESBL producing *E. coli* showed a high degree of (86.1%) resistance to both cefuroxime and cefotaxime, followed by ceftazidime. According to Pootong *et al*. (2018), the prevalence of ampicillin resistance was the highest (89.1%), followed by cefotaxime (40.3%), ceftazidime 15.1%, and ESBL antibiotic producing bacteria (Ceftazidime-Clavulantae, Cefotaxime-Clavulantae) 38.7% of the outcomes were different from what was expected in the present study. Antibiotic resistance has been found in several studies due to widespread availability, uncontrolled usage, and high concentrations of antibiotics in animal feed (Nji *et al*., 2021, Pormohammad *et al*., 2019).

Detection of biofilm formation by Congo red agar (CRA)

The capacity of bacteria to build biofilms is one of the most important indicators of their pathogenicity and antibiotic resistance. According to the results in the research, *E. coli* biofilms were detected in 71% of the 100 isolates tested using Congo red agar. A dry or glossy black colony was the clear sign of a bacteria producing biofilm (Fig. 3), while non-membrane-forming *E. coli* isolates were found to be bright pink, red, or wine-colored colonies. Mohsenzadeh *et al*. (2021) noted that out of 100 *E. coli* and *P. aeruginosa* isolates, 38 (38 %) demonstrated a biofilm-positive phenotype under optimum circumstances in MTPA and isolates were further categorised as strong, moderate, weak, and no biofilm on Congo red agar The present study showed the number of UPEC that formed biofilm in vitro was 71%, which was comparable to the studies that were carried out by previous studies of Subramanian *et al*. (2012) Sharma *et al*. (2009) and Suman *et al*. 2007 that discovered rates of biofilm development of *E. coli* isolates as 63% , 67.5%, and 92.0% respectively. *In vitro*, over 50 drug-resistant UPEC strains were investigated for their ability to form biofilms by forming slime on CRA. It was shown that 68% of the 50 *E. coli* isolates of UPEC infections (34 out of 50) had biofilms (Javed *et al*., 2021).

Phylogenetic groups and subgroups of *E. coli* isolates

Polymerase chain reaction was used to determine the presence of the *chu*A gene, *yja*A gene, and TspE4.C2 DNA fragment. Among UTI patients, the results of 100 *E. coli* isolates belonging to phylogroup B2 (91, 91 %) and subgroup B23 (75, 75 %) were the most prevalent (Fig. 4). *E. coli* isolates belonged to extraintestinal phylogenetic group D (6, 6%) and subtyping D_1 (4.4%) (Table 2). The PCR-based phylogenetic typing established by (Clermont *et al*., 2000) efficiently screens for ExPEC based on the presence or lack of this specific marker sit. *E. coli isolates* belonging to one of the seven phylogenetic subgroups (A0, A1, B1, B22, B23, D1 and D2) showed on 1.5% Agarose gel electrophoresis for *chu*A amplicon (288bp) , *yja*A amplicon (211 bp) and TspE4C2 amplicon (152 bp). Isolates that belonged to the phylogenetic groups B2 (91, 91 %) and B23 (75, 75 %) exhibited a high level of resistance to the antibiotics

that were tested in comparison to other groups Table 2 . Phylogenetic group B2 was the most common in the present investigation. Several other researchers were in consistent with the present conclusion Lindblom (2020), Javed *et al*., 2021). The investigation research study found that 53% of isolates belonged to phylogroup B2 Lin *et al*., 2022). Other studies showed that human pathogenic (ExPEC) predominantly belonged to B2 and D groups (Giufre *et al*., 2021; Mahmoud *et al*., 2020), which are also considered to be more virulent and more associated with infections.

Table 2. Distribution of antibiotic resistant *E. coli* isolates in various (A) phylogenetic groups and (B) phylogenetic groups subtyping

A

Isolates No. (%)	Phylogentic group
1	A
2	B1
91	B2
6	D
100%	

B

Isolates No.%	Subtyping
0	A_0
1	A_1
2	B_1
16	$B2_2$
75	$B2_3$
4	D_1
2	D_2
100%	

Table 3. Distribution of Biofilm producing in various (A) phylogenetic groups (A, B1, B2, D) and (B) phylogenetic groups subtyping (A0, A1, B1, B22, B23, D1, D2) of *E. coli* Isolates

A

Phylogroups	Biofilm formation No. of Isolates	%
A	0	0
B1	1	1.4
B2	67	94.4
D	3	4.2
Total	71	100

B

Subgroup/Biofilm	Biofilm formation No. of Isolates	%
A0	0	0
A1	0	0
B1	1	1.4
$B2_2$	11	15.5
$B2_3$	56	78.9
D1	2	2.8
D2	1	1.4
Total	71	100

Association between phylogenetic group and biofilm formation of *E. coli* isolates

The production of bacterial biofilms is a critical virulence mechanism that promotes colonisation and drug resistance. Both B2 and D contain all of the biofilm-

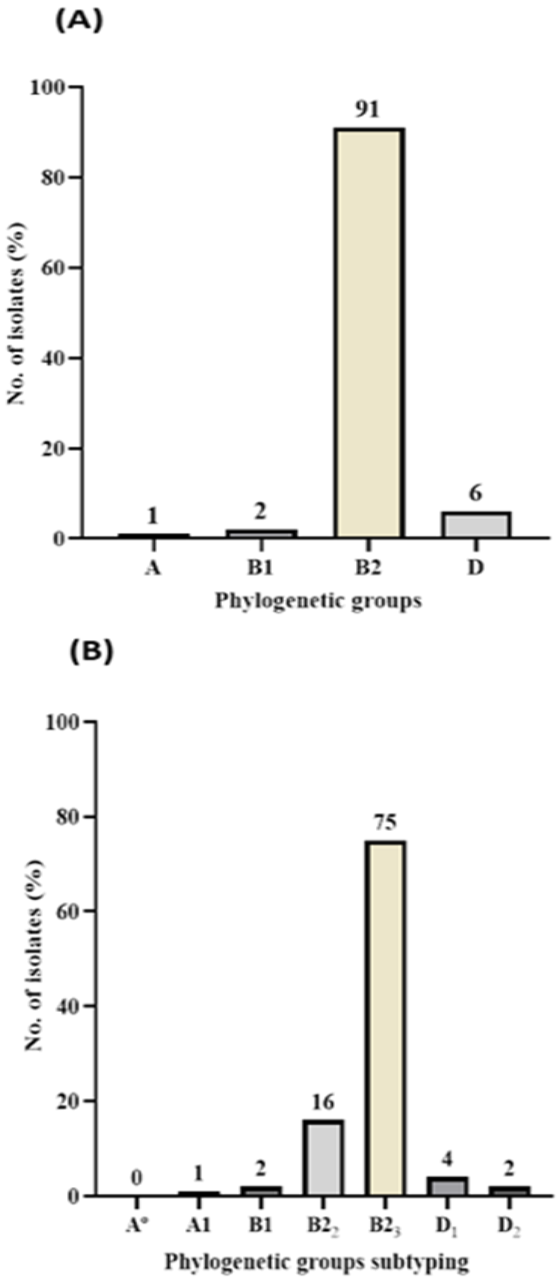

Fig. 4. *Distribution of the E. coli isolates among the UTIs. Distribution of the E. coli isolates (A) according to phylogenetic groups (A, B1, B2, and D) and (B) subtyping (A0/A1, B1, $B2_2$/$B2_3$, and D_1/D_2)*

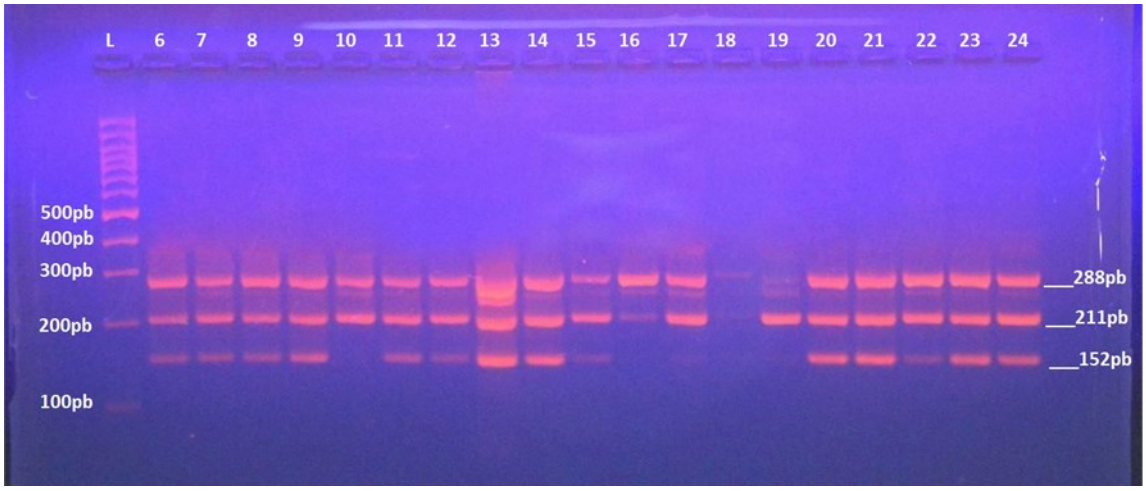

Fig. 5. *Representative agarose gel electrophoresis of E. coli genes used to classify E. coli into various phylogenetic groups (Lane M is 1kb DNA ladder, and lanes 6 to 24 are amplified PCR products with the following Escherichia coli phylogenetic grouping genes; chu A (288 bp), yja A (211 bp) and TspE4C2 (152 bp). Samples were amplified on 1.5% agarose gel and isolates are labeled at the top of this figure*

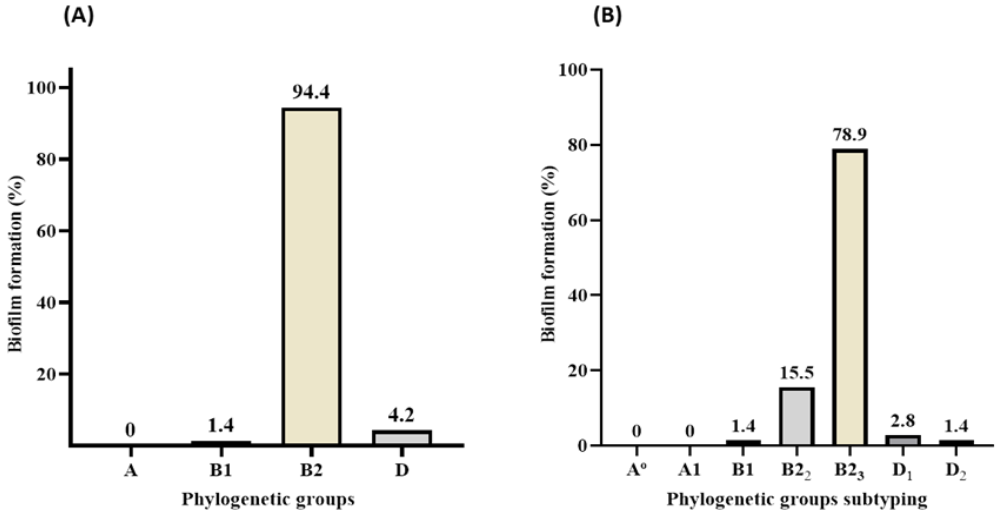

Fig. 6. *Biofilm producing ability among different (A) phylogenetic groups (A, B1, B2, and D) and (B) subtyping (A0/A1, $B2_2$/$B2_3$, B1 and D_1/D_2) of E. coli isolates*

related genes present in their genome. FimH has been found in several *E. coli* phylogroup studies (Olowe *et al*., 2019). In present research, it was observed that *E. coli* isolates from phylogenetic group B2 and subgroup $B2_3$ were more likely to generate biofilms than isolates belonging to phylogenetic groups A, B_1, and D/ subtyping A_0/A_1, B_1 and D_1/D2 (Table3). Distribution of Biofilm production in various phylogenetic groups (A, B1, B2, and D) and phylogenetic groups subtyping (A0, A1, B1, B22, B23, D1, and D2) of *E. coli* from phylogenetic group B2 and subgroup B23 were found to have greater biofilm producing ability than isolates from phylogenetic groups A, B1, and D (Fig. 6). In accordance with several studies such as the one conducted by Javed *et al*. (2021), majority of Uropathogenic *Escherichia coli* (UPEC) strains cause recurrent UTI infections that were discovered in phylogenetic groups B2 formed strong biofilm, while phylogenetic group D included strong and moderate biofilm formers. When all of the facts are taken into consideration among the members of groups B1 and A, the biofilm was considerably weaker because of the availability of pathogenicity islands and expression of additional virulence genes such as adhesion factors, cell surface hydrophobicity, siderophore and poison production, etc. Nielson *et al.* (2018) found a similar result for extraintestinal and commensal *E. coli* isolates that cause UTI infections . There was no big variation between groups B2 and B1 or D, so a higher prevalence of these isolates produced moderate or higher biofilms at 43.5%, 41.3%, and 27.9 %, respectively, among the isolates examined. The majority of group A isolates produced negligible biofilms (55.6 %). The present findings indicate biofilm production was related to *E. coli* strains phylogenetic group B2 and subgroup B22.

Conclusion

The present investigation found that ESBL-producing *E. coli* isolates were prevalent in individuals with UTIs. *E. coli* isolates analyzed in this study were resistant to Aztreonam, Ceftriaxone, Cefpodoxime, Ceftazidime, Ceftazidime-Clavulantae, Cefotaxime, and Cefotaxime-Clavulantae when tested for antibiotic resistance. The present findings revealed that the most common phylogenetic group of *E. coli* found at Al-Hillah hospitals was B2, with the next most common being D, and the smallest were B1 and A.

ACKNOWLEDGEMENTS

I want to extend my sincere thanks to everyone who helped me collect samples from the medical staff at Marjan Medical City and complete the research.

Conflict of interest

The authors declare that they have no conflict of interest.

Ethical Approval

After getting the go-ahead from the appropriate authorities, everyone involved gave the project the green light. The details were recorded and saved as name, gender, age, and date of infection for each patient as well as the onset and progression of any chronic illnesses

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