Investigation of virulence factors and their relationship with antimicrobial resistance among uropathogenic *Escherichia coli* isolates identified from patients in Basrah city, Iraq

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Summary

Uropathogenic *Escherichia coli* (UPEC) is one of the main causes of urinary tract infections (UTIs). We aimed to investigate the antimicrobial resistance (AMR) pattern, the frequency of some virulence genes (VGs), and the association of AMR with VGs.

A total of 300 urine samples were collected from patients suspected to have UTI. The samples were examined by biochemical and microbiological methods and VITEK2 compact system to identify the bacterial infectious agents. The antimicrobial resistance pattern and virulence genes

(*papAH, papC, papEF, papG, fimH*, and *fyuA*) profile of UPEC isolates were investigated and the relationship between these traits was evaluated by statistical methods.

Among these samples, 201 (67%) exhibited a positive growth on culture media. *E. coli* was isolated from 60 (29.85 %) specimens followed by *Klebsiella pneumoniae* 42 (20.90%), *Staphylococcus aureus* 38 (18.9%), *Enterobacter* spp 29 (14.43%), *Pseudomonas aeruginosa* 10 (4.98%), *Proteus mirabilis* 15 (7.46%), others about 7 (3.48 %) isolates. Antibiogram results of 15 antibiotics examined showed that all *E. coli* isolates were multidrug-resistant (MDR). The commonest antimicrobial resistance was observed against Streptomycin (100%), Kanamycin (98.3%), and Ampicillin (96.7%). The most sensitive agents were Meropenem (96.4%), Nitrofurantoin (93.4%), and Imipenem (85%). VGs detected among UPEC isolates were *fimH* (88.3%), *papAH* (85%), papC (85%), *papG* (80%), *fyuA* (80%), and *papEF* (60%).These results alleged no strong correlation between VGs and AMR in *E. coli* strains.

Based on the results of the present study, virulence genes, and antimicrobial resistance are independent properties and can transfer to other bacteria separately.Further studies are needed to better understand the relationship between different virulence factors (VFs) and AMR at a molecular level, as most UPEC isolates express several VFs and AMR simultaneously.

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Key words
Uropathogenic Escherichia coli; Antimicrobial Resistance; Virulence genes; Urinary tract infection

Introduction

Urinary tract infection (UTI) is an important infectious disease in humans. Previous studies have reported that UTI can be caused by many pathogens such as *Escherichia coli, Klebsiella, Proteus, Pseudomonas*, and other microorganisms.[1] *E. coli*, the main cause of UTI, is a part of the normal flora of healthy individuals but some strains may lead to disease in the intestine or some other organs. Uropathogenic *E. coli* (UPEC) strains have acquired virulence properties that enable them to cause disease. Pathogenic *E. coli* strains are generally classified into three groups of diarrheagenic, uropathogenic and strains causing other extra-intestinal manifestations, including sepsis/meningitis.[2]

To form a UTI, *E. coli* needs to overcome several defense lines. Virulence factors (VFs) are molecules produced by bacteria that enable the microorganisms to thrive within host species, and increase the ability of microbes to cause disease.[3] Virulence genes (VGs) of UPEC strains are transferred by plasmids or localized on chromosomal gene clusters, called "pathogenicity islands".[4]

Some of the major VFs which found in UPEC are ad-

hesins, protectins, toxins, and iron-acquisition systems.[5] More than ten different pilus systems are recognized in UPEC strains; however, the most common and best characterized are type 1 and P pili encoded by the *fim* and *pap* operons, respectively.[6]

The second common VG of UPEC is *papC* which plays an important role in producing pylonephritis caused by ascending UPEC strains. P fimbriae are heteropolymeric fibers composed of different protein subunits including Pap C, Pap A, Pap H, Pap K, Pap E, and Pap F.[7]

Several iron-acquisition systems have been identified in pathogenic *E. coli*. Yersiniabactin is associated with a siderophore system in which low mass molecules have high affinities for iron absorption, and are important in pathogenic bacteria. Yersiniabactin could solubilize the metal bounds in host binding proteins and transport iron to bacteria because of their high affinity to iron. The expression of yersiniabactin is regulated by *fyuA* gene.[8]

Antimicrobial resistance (AMR) genes are typically acquired through mobile genetic elements (MGE) including plasmids, insertion sequence elements, transposons, and integrons.[9]

Many MGEs, especially plasmids, are transferred between different members of the *Enterobacteriaceae* enabling the spread of the resistance genes.[10] MGE scan also encode VFs, and there may be an interplay between virulence and AMR.[9] AMR is rapidly increasing and has become a global problem. Resistance development and appearance of multidrug-resistant (MDR) strains in UTIs may be due to frequent use of antimicrobial agents usually with broad spectrum, hospitalization, anomalies in the urinary tract system, age, catheterization, and recurrent UTIs.[11]

Several studies have shown that resistant *E. coli* strains tend to be less pathogenic than susceptible isolates; however, some studies reported opposite results.[12,13] Furthermore, it remains unclear whether virulence and antimicrobial resistance are co-associated factors or independent. In this study, we investigated the frequency of some VGs and resistance against antibiotics, then their relationships were determined.

Materials and Methods

Sample Collection

The current study was conducted at the AL Zubair Hospital and Dar AL Shifaa Investment Hospital in Basra; Iraq, from November 2020 to March 2021. The urine samples were collected from patients referred to AL Zubair hospital and Dar AL Shifaa Investment hospital in Basra, Iraq. A total of 300 urine specimens were collected, and then the demographical data such as name, age, gender, address and clinical manifestation (cystitis, pyelonephritis) were recorded by physicians. The samples of patients who had taken any medicine in the past three days were excluded from the study. Midstream urine was collected from suspected patients and sent to the laboratory for further analysis.

Isolation and identification of microorganisms

Urine samples were analyzed and then cultured on blood agar (Merck, Germany) and MacConkey agar (Merck, Germany). Cultured samples were incubated overnight at 37°C. If the growth count was less than 10^3 CFU/mL or when growth of two or more species were detected, the samples were considered negative or contaminated, respectively. Urinary infection was positive by the number of $\geq 10^5$ CFU/mL. Grown bacteria were identified using conventional morphological and biochemical tests including Gram staining, culture on Eosin Methylene Blue (Merck, Germany), Triple Sugar Iron agar (TSI)(Merck, Germany), Methyl Red-Voges-Proskauer (MR-VP)(Merck, Germany), and Simmons Citrate agar (Merck, Germany). Final confirmation was performed using VITEK2 compact system (Biomerieux , USA).

Antimicrobial Susceptibility Testing

All *E. coli* isolates were subjected to antibiotic susceptibility analysis using the Kirby-Bauer disc diffusion method. The selection of antibiotic discs was done according to the guidelines recommended by the Clinical and Laboratory Standard Institute (CLSI, 2020).[14] Antibiotic discs (Liofilchem, Italy) used, included streptomycin (10 μg), kanamycin (30 μg), ciprofloxacin (5 μg), amikacin (30 μg), amoxicillin-clavulanate (30 μg), tetracycline (30 μg), meropenem (10 μg), ampicillin (25 μg), cefotaxime (30 μg), trimthoprim-sulphametoxazole (25 μg), nitrofuranation (300 μg), ceftazidime (30 μg), and imipenem (10 μg).

Genomic DNA Extraction

Genomic DNA of *E. coli* isolates was extracted using Wizard® Genomic DNA extraction kit (Promega, USA) according to the instructions. The extracted DNA was stored at -20°C until use.

Multiplex PCR

The presence of *papAH, papC, papEF, papG, fimH*, and *fyuA* genes was evaluated by multiplex PCR. The primer sequences and reactions were performed according to protocols described by Johnson and Stell.[15] Amplification was done in a 25-μL reaction mixture containing 12.5 μL PCR master mix (Promega; USA),

5µL DNA template, 0.6 µM of each primer and distilled water up to 25µL. PCR amplifications consisted of primary denaturation at 94°C for 5 min, 35 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min and extension at 72°C for 1 min and finally an extension at 72°C for 7 min in a thermal cycler (Applied Bio System USA).

Statistical Analysis

Relationship between VFs and antimicrobial resistance was evaluated using Spearman's correlation coefficient matrix. Results were shown using a correlogram drawn by R software (version 4.1.3). Correlations with a $p < 0.001$ were considered significant. A dendrogram was drawn according to UPGMA (unweighted pair group method with averaging) supported by the Numerical Taxonomy and Multivariate Analysis System (NTSYS) package version 2.02pc to evaluate the similarity between isolates based on VGs profile and antimicrobial resistance pattern of UPEC isolates.

Results

Profile of bacteria isolated from patients with UTI

In the present study, 300 urine samples were collected from patients suspected to have UTI based on clinical manifestations. Among these, 201 (67%) exhibited growth of uropathogenic bacteria, while 99 (33%) showed no growth. Out of 201 detected isolates, 38 (18.9%) and 163 (81.1%) were Gram-positive, and Gram-negative bacteria, respectively. The positive cultures for women and men were 152 (75.62%) and 49 (24.37%), respectively, which was statistically significant ($p < 0.05$). These identified bacteria belonged to different genera. Among Gram negatives, 60 (29.85%), 42 (20.90%), 29 (14.43%), 15 (7.46%), and 10 (4.98%) urine specimens were positive for *E. coli, Klebsiella pneumoniae, Enterobacter* sp., *Proteus mirabilis,* and *Pseudomonas aeruginosa*, respectively. The other Gram negatives were about seven (3.48 %). *Staphylococcus aureus*, a Gram positive pathogen, was isolated from 38 urine samples (Fig 1). The samples which showed no bacterial growth or growth below 10^5cfu/mL were excluded from the study. Symptoms of these patients could be attributed to other causes outside the target of this study. Therefore, we found *E. coli* as the predominant etiology of UTI.

Antimicrobial Susceptibility Determination

All UPEC isolates were subjected to antimicrobial susceptibility testing according to the CLSI (2020) guidelines. As shown in Fig 2, antimicrobial resistance was determined, in receding frequency, against streptomycin (100%), ampicillin (95%), ceftazidime (93.3%), cefotaxime (86.7%), trimethoprim-sulphametoxazole (78.3%), amoxicillin-clavulanate (63.3%), nalidixic acid (58.3%), ciprofloxacin (53.3%), kanamycin (40%), gentamycin (36.7%), amikacin (26.7%), imipenen (15%), nitrofurantoin (6.6%), meropenem (3.3%) (Fig 2).

Distribution of VGs

The PCR results showed that 96% of the UPEC isolates

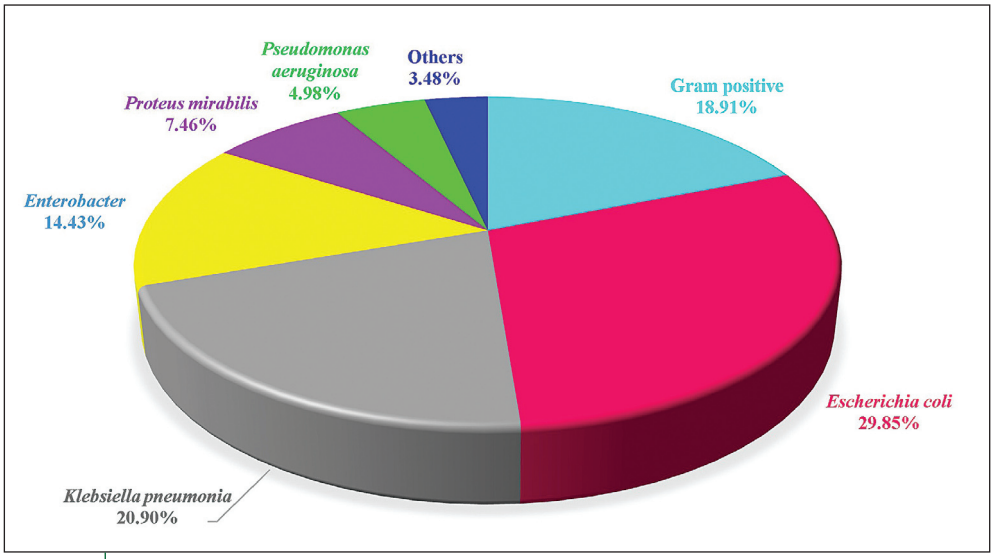


Figure 1 Profile of bacteria isolated from patient with urinary tract infection.

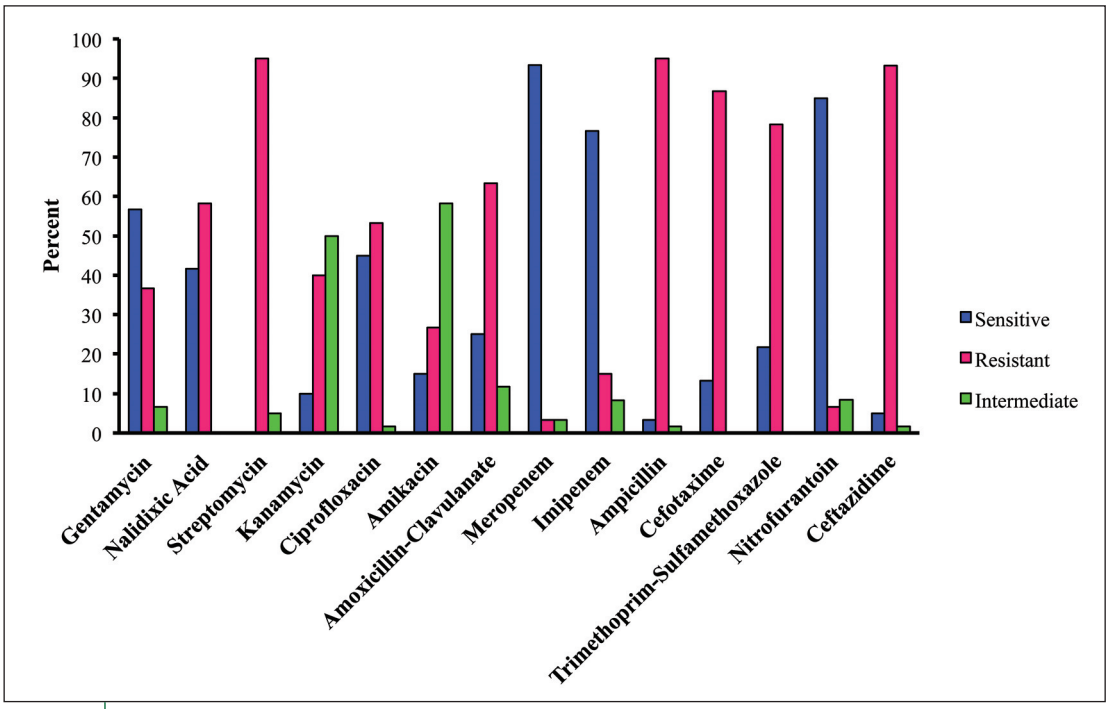


Figure 2 Antibiotic susceptibility pattern of uropathogenic *Escherichia coli* isolates.

had at least one of the investigated VGs. The highest and lowest frequencies were related to the *fimH* (88.3%) and the *papEF* (60%) isolates. The genes of *papC, papAH, papG*, and *fyuA* were detected in 51 (85%), 51 (85%), 48 (80%), and 48 (80%) of UPEC isolates, respectively.

Correlation between antimicrobial resistance and VGs

Spearman's correlation coefficient matrix was performed to determine the relationship of antimicrobial resistances, VGs, and antimicrobial resistances with VGs.The strongest associations were detected among resistance to: nalidixic acid and gentamycin, ciprofloxacin and gentamycin, ciprofloxacin and nalidixic acid, and amoxicillin-clavulanic acid and kanamycin. *pap* genes showed a relationship with each other (Fig 3).

Cluster analysis

The drawn dendrogram showed 35 (58%) isolates located in one major cluster due to similarity in genetic background and antimicrobial resistance pattern (Coefficient of 0.8) (Fig 4).

Discussion

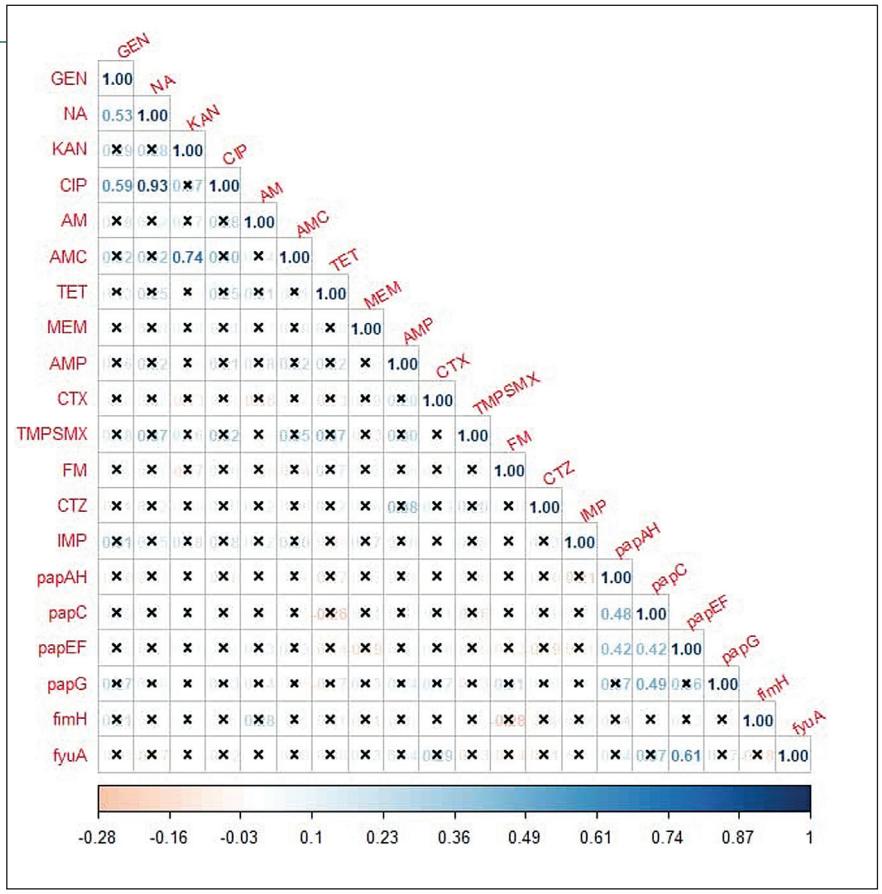
Based on our results, UPEC is one of the major causative agents of UTIs in Basrah city, Iraq. In the present study, the frequency of UPEC isolates was higher in females than in males. It could be due to hormonal and anatomical differences of the urinary tract in females.

Findings of the present study revealed a high rate of resistance to streptomycin, kanamycin, and ampicillin. Studies from other countries also reported a high frequency of AMR against UPEC isolates.[12,16] We found that 63.3% of the UPEC isolates were resistant to amoxicillin-clavulanic acid. In Mexico, UPEC strains related to outpatients with uncomplicated UTI showed 19.6% resistance against amoxicillin-clavulanic acid.[17] Another study on community-acquired UTIs in Italy reported 18.2% resistance against amoxicillin-clavulanate in UPEC isolates.[18]

With respect to the findings of the present study, UPEC isolates showed high sensitivity to meropenem, nitrofurantoin and imipenem, which was in accordance with the results of other studies.[19-20] Nitrofurantoin is prescribed only for the treatment of uncomplicated UTIs, because it cannot reach an appropriate level in the bloodstream. Nitrofurantoin is not recommended for complicated UTI or systemic involvements; however, due to increased resistance against sulfamethoxazole-trimethoprim and quinolones in UPEC strains, the rational use of nitrofurantoin has been recommended in cases such as re-infection or prophylaxis of recurrent uncomplicated UTIs.[21-22]

Figure 3

Correlogram generated according to the relationship between the presence of UPEC virulence genes and antimicrobial susceptibility pattern. The relationships with p> 0.05 are marked with a cross (x). GEN: gentamycin; NA: nalidixic acid; KAN: kanamycin; CIP: ciprofloxacin; TET: tetracycline; AM: amikacin, AMC: amoxicillin-clavulanate, TET: tetracycline, MEM: meropenem, AMP: ampicillin, cefotaxime, TMP-SMX: trimthoprim-sulphametoxazole, FM: nitrofuranation, CTZ: ceftazidime, and IMP: imipenem.



Detection of VFs can improve our knowledge about the pathogenic processes of UTI and reduce complications such as kidney failure. In the present study, we determined the frequency of six important VGs among UPEC isolates. We deployed a molecular method to explore the association between some VGs and AMR in UPEC strains isolated from patients with UTI in Basrah city, Iraq. UPEC isolates carry different Vgs and are diverse due to the presence of MGEs such as plasmids, transposons, pathogenic islands (PAIs), and bacteriophages. These genetic elements may carry AMR genes in addition to Vgs. It has been reported that some plasmids belonging to the IncF incompatibility group, carry both virulence and antibiotic resistance genes.[12]

Type 1 and P fimbriae are frequent among UPEC strains isolated from cystitis and pyelonephritis, respectively.[23] In the present study, *fimH* which encodes the type 1 fimbria was found in almost (88.3%) UPEC isolates. Other studies reported a lower frequency of *fimH* such as that reported by Paniagua-Contreras *et al* (2017) in Mexico with a prevalence rate of 61.3%.[24] Tiba *et al* conducted a study on the VGs of UPEC strains isolated from patients with cystitis.[25] The highest frequency rates of VFs were related to *fimH* (97.5%) and *papC* (32.7%). In *E. coli* strains isolated from Romanian adults with UTI, the incidence rate of *fimH* and *papC* was reported at 86% and 36%, respectively.[26] In our study, both *papC* and *papAH* were found in 85% of UPEC isolates.

The results showed that the UPEC isolates carried these six VFs with high frequencies. These results demonstrated no strong correlation between VGs and AMR in *E. coli* strains. A strong association between *pap* genes indicates that these genes are located in a gene cluster.

Johnson *et al* believe that AMR and virulence do not usually co-evolve and most multidrug resistant isolates are less virulent than susceptible ones.[13] However, some researchers reported a positive association between virulence traits including *fimH* receptors with AMR.[27,28] There are many contradictions in this regard. To sum up, UPEC strains differ in their pathogenic potential and susceptibility to antimicrobial drugs in different patient populations, a point that should be considered when guidelines are developed for the management of UTI. Regular evaluations and formulation of antibiotic use policy are essential in

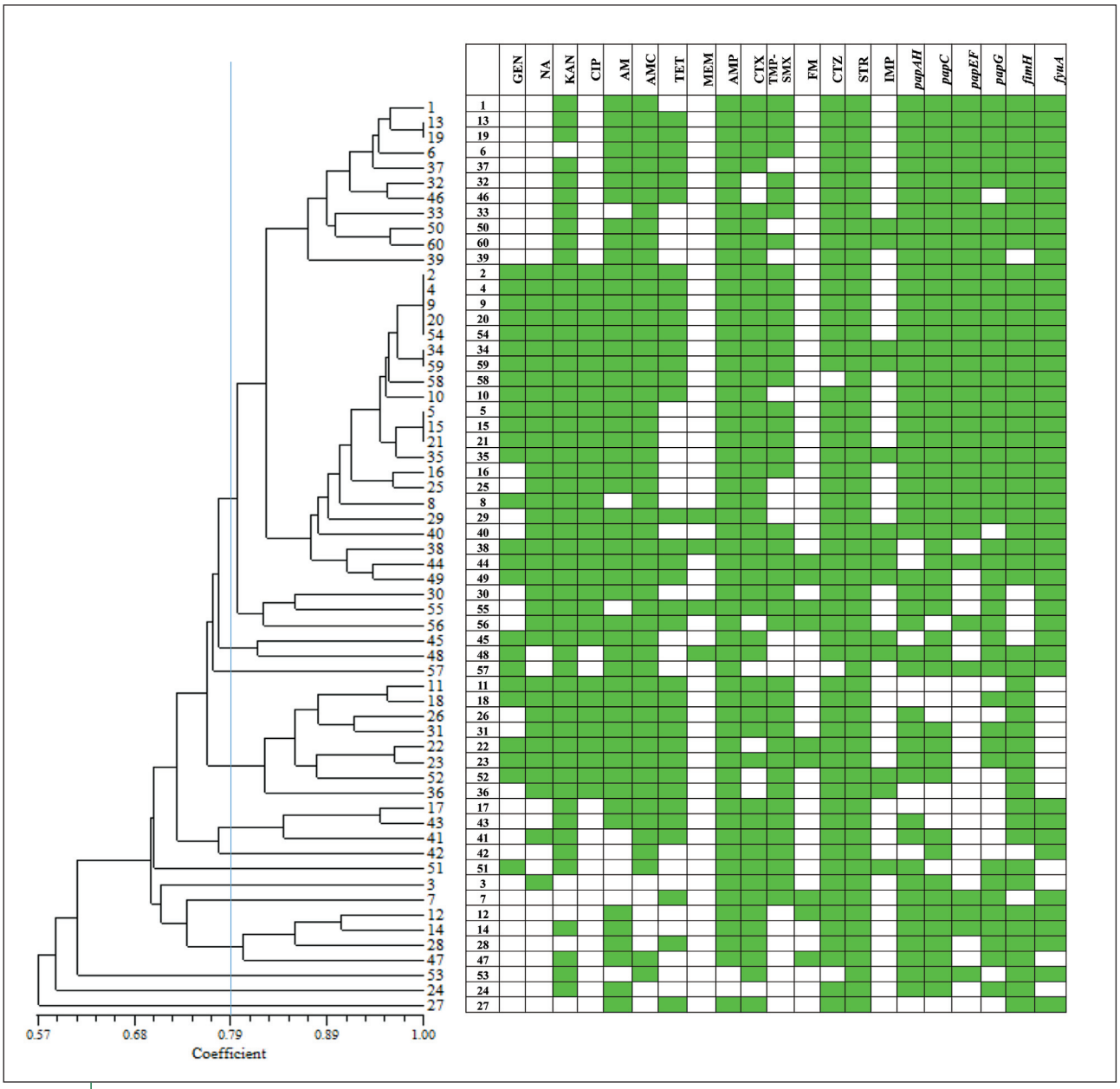


Figure 4 UPGMA (Unweighted pair-group method with arithmetic clustering) dendrogram according to data from antimicrobial resistance phenotype and virulence genes of uropathogenic *Escherichia coli* strains. Positive genes or antimicrobial resistance were shown with green color, while negative genes or antimicrobial susceptibility were shown with white color. GEN: gentamycin; NA: nalidixic acid; KAN: kanamycin; CIP: ciprofloxacin; TET: tetracycline; AM: amikacin, AMC: amoxicillin-clavulanate, TET: tetracycline, MEM: meropenem, AMP: ampicillin, cefotaxime, TMP-SMX: trimthoprim-sulphametoxazole, FM: nitrofuranation, CTZ: ceftazidime, and IMP: imipenem.

managing the transmission and acquisition of AMR. Further studies are necessary to better understand the relationship between different VFs and AMR at a molecular level as most UPEC isolates simultaneously express several AMR or VFs. However, based on the results of this study VGs and antimicrobial resistance are independent properties and can transfer to other bacteria separately.

Conclusion

E. coli is one of the main causative agents of UTI. The results showed that UPEC isolates harbored different VFs. Moreover, we did not find a strong connection between VFs and AMR in UPEC isolates. Further studies are needed to better understand the relationship between different VFs and AMR at a molecular

level, as the most UPEC isolates simultaneously express several VFs and AMR.

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Ethics statement
The study was approved by Ethics Committee of Shahid Chamran University of Ahvaz.

Conflicts of Interest
The authors declare that they have no competing interests.

Authors' contributions
All authors contributed to the design of the experiment. SER and YYYA designed and supervised the research study. HA carried out the experiments. MRA participated in the design of the study and data analysis. All authors read and approved the final manuscript.

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Περίληψη

Investigation of virulence factors and their relationship with antimicrobial resistance among uropathogenic *Escherichia coli* isolates identified from patients in Basrah city, Iraq

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Το ουροπαθογόνο στελέχη *Escherichia coli* (UPEC) είναι μια από τις κύριες αιτίες ουρολοιμώξεων (UTIs). Στόχος της παρούσας μελέτης ήταν η διερεύνηση της αντιμικροβιακής αντοχής (AMR), η συχνότητα ορισμένων γονιδίων λοιμογόνου δράσης (VGs) και η τυχόν συσχέτιση τους. Συνολικά συλλέχθηκαν 300 δείγματα ούρων από ασθενείς με υποψία ουρολοίμωξης. Τα δείγματα εξετάστηκαν με τις κλασικές βιοχημικές και μικροβιολογικές μεθόδους, ενώ η ταυτοποίηση και ο έλεγχος ευαισθησίας των απομονούμενων μικροοργανισμών έγινε με το ημιαυτοποιημενο σύστημα VITEK2 (bioMerieux). Στη συνέχεια, προσδιορίστηκε το προφίλ γονιδίων λοιμογόνου δράσης (*papAH, papC, papEF, papG, fimH,* and *fyuA*) των απομονούμενων UPEC και έγινε διερεύνιση τυχόν συσχέτισή τους με το αντίστοιχο προφίλ της αντιμικροβιακής αντοχής με στατιστικές μεθόδους. Μεταξύ των δειγμάτων, 201 (67%) έδωσαν θετικό αποτέλεσμα στην καλλιέργεια. Ο μικροοργανισμός *E. coli* απομονώθηκε από 60 (29,85 %) δείγματα, ακολουθούμενος από τους μικροοργανισμούς *Klebsiella pneumoniae* 42 (20,90%), *Staphylococcus aureus* 38 (18,9%), *Enterobacter* spp. 29 (14,43%), *Pseudomonas aeruginosa* 10 (4.98%), *Proteus mirabilis* 15 (7.46%) και λοιποί μικροοργανισμοί 7 (3.48 %). Τα αποτελέσματα αντιβιογράμματος για 15 αντιβιοτικών που εξετάστηκαν έδειξαν ότι όλα τα απομονωμένα στελέχη *E. coli* ήταν πολυανθεκτικά (MDR). Η συνηθέστερη αντιμικροβιακή αντοχή παρατηρήθηκε έναντι στρεπτομυκίνης (100%), καναμυκίνης (98,3%) και αμπικιλλίνης (96,7%). Τα μεγαλύτερα ποσοστά ευαισθησίας παρατηρήθηκε σε μεροπενέμη (96,4%), νιτροφουραντοΐνη (93,4%) και ιμιπενέμη (85%). Τα VGs που ανιχνεύθηκαν μεταξύ των απομονώσεων UPEC ήταν: *fimH* (88,3%), *papAH* (85%), *papC* (85%), *papG* (80%), *fyuA* (80%) και *papEF* (60%). Η στατιστική ανάλυση έδειξε ότι δεν υπάρχει ισχυρή συσχέτιση μεταξύ VGs και AMR στα στελέχη *E. coli* της μελέτης. Συμπερασματικά, τα γονίδια λοιμογόνου δράσης και η αντιμικροβιακή αντοχή είναι ανεξάρτητες ιδιότητες και μπορούν να μεταφερθούν χωριστά σε άλλα βακτήρια. Απαιτούνται περαιτέρω μελέτες για την καλύτερη κατανόηση της σχέσης μεταξύ διαφορετικών παραγόντων λοιμογόνου δράσης (VFs) και AMR σε μοριακό επίπεδο, καθώς τα περισσότερα απομωνούμενα στελέχη UPEC εκφράζουν ταυτόχρονα πολλά VFs και πολυαντοχή.

Λέξεις κλειδιά
ουροπαθογόνο Escherichia coli, UPEC, αντιμικροβιακή αντοχή, γονίδια λοιμογόνου δράσης, λοίμωξη του ουροποιητικού συστήματος

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