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Original article

Resistance and Virulence Factor Determinants of Carbapenem-Resistant *Escherichia coli* Clinical Isolates in Three Hospitals in Tehran, IR Iran

Farshad Nojoomi[1], Abdolmajid Ghasemian[1*]

[1]Department of Microbiology, Department of Medicine, AJA University of Medical Sciences, Tehran, IR Iran

*Corresponding author: Abdolmajid Ghasemian, Department of Microbiology, Faculty of Medicine, AJA University of Medical Sciences, Tehran, IR Iran, E-mail: majidghasemian86@gmail.com, Tel: +989394514860. Fax: +982182334674

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Abstract

Background: *Escherichia coli* (*E. coli*) strains are among predominant agents causing nosocomial and community acquired infections. The majority of strains encode numerous virulence factors including fimbrial adhesions, secretory proteins and toxins, siderophores, and capsule. This study aimed to investigate the prevalence rate of virulence encoding genes and carbapenem resistance-encoding genes among imipenem-resistant *E. coli* isolates collected from patients hospitalized in Tehran, IR Iran.

Materials and Methods: In this cross-sectional study (April 2015-December 2017), 50 non-duplicated carbapenem-resistant *E. coli* isolates were collected from clinical specimens (stool, urine, blood, and wound) of hospitalized patients in three hospitals in Tehran, Iran. The antibiotic susceptibility profile was determined against 15 antibiotics on Mueller Hinton Agar (MHA) as per CLSI guidelines version 2016. The PCR was used to detect virulence and antibiotic resistance encoding genes.

Results: From a total of 50 carbapenem-resistant *E. coli* isolates, the highest resistance rate was observed to ceftazidime (100%), tetracycline (88%), amoxicillin (100%), sulfonamide (60%), and the least resistance rate was observed against amikacin (14%), gentamicin (22%), and fosfomycin (0%). The genes mediating resistance were as follows: beta-lactams OXA-48 (8%), IMP (16%), VIM (0%), NDM-1 (0%), *fosA3* (0%), quinolones (*qnrA* 48%), and colistin *mcr-1*(0%). Furthermore, the prevalence rates of *of fimA, hlyA, cnf1, vat, pic, crl,* and *papH* were 88, 36, 28, 10, 12, 54, and 88%, respectively.

Conclusion: In this study, all imipenem-resistant *E. coli* isolates were susceptible to fosfomycin, and all were *fosA3* negative. Among carbapenemase genes, IMP and OXA-48 type enzymes associated with higher MIC levels (8 to 32 $\mu g.mL^{-1}$) were detected. In this study, data suggest the role of these carbapenemases in resistance to carbapenems. Furthermore, the presence of multiple drug resistant strains encoding adhesive and secretory virulence factors is a concern for the infections treatment.

Keywords: *Escherichia coli*, Virulence, Carbapenemases

1. Background

Escherichia coli strains or pathotypes are genetically diverse groups causing several types of infections. These strains encode a number of adhesions mediating their persistence and colonization in epithelial cells, resulted in destroying the host immune and defense mechanisms and initiating extra-intestinal infections (1). In addition to fimbrial adhesions, toxins, iron uptaking siderophores, and polysaccharide capsule are also involved in the pathogenesis of the isolates. Toxin production following the colonization of *E.coli* strains may induce inflammatory responses (2-3). Alpha hemolysin (HlyA) and cytotoxic necrotizing factor Type 1 (CNF1) are two known toxins which have been demonstrated to cause direct cytotoxicity in host tissues. Three different toxins from the SPATE (serine protease auto-transporters of Enterobacteriaceae) family have been identified in *E. coli* strains from pyelonephritis, Sat (secretory protein), Pic (protease leading to colonization), Vat (vacuolating auto-transporters toxin), and Tsh (the temperature sensitive hemagglutination), all of which are widespread in UPEC but not in commensal strains (4-5). Furthermore, *E. coli* adhesions such as Type 1 fimbriae and pili are important for bacterial colonization (6).

Drug-resistant Enterobacteriacea, especially those producing extended-spectrum b-lactamases (ESBLs), are typically treated with carbapenems. Increase in imipenem resistance among these species have led to the difficulties in infection eradication (7). Several carbapenemase-encoding genes such as bla_{IMP}, bla_{VIM}, bla_{OXA-48}, bla_{NDM1}, and bla_{GES} have been participated in carbapenem resistance (8-9). In Iran, previous studies have detected some of these genes with various imipenem minimum inhibitory concentrations (MICs); however, the relationship between carbapenemases and virulence factors among imipenem-resistant *E. coli* strains has not been fully elucidated.

2. Objective

This study aimed to investigate the prevalence rate of virulence encoding genes and carbapenem resistance-encoding genes among imipenem-resistant *E. coli* isolates collected from patients hospitalized in Tehran, IR Iran. 3. Materials and Methods.

3. Materials and methods

3.1. Bacterial isolates

This cross-sectional survey was performed from April 2015 to December 2017. A total of 50 carbapenem-resistant *E. coli* isolates were isolated from patients hospitalized in intensive care units (ICU) (31/50) and in infectious disease (9/50), surgery (7), and internal (3) wards. The specimens included stool, urine, blood, and wound from 3 hospitals in Tehran, Iran. The ages of patients ranged from 49 to 73 years, among them 39 cases were female, and 11 cases were male. The *E. coli* isolates were identified by employing conventional

biochemical tests and stored in Trypticase Soy Broth (TSB) medium containing 30% glycerol at - 20°C for further studies.

3.2. Antibiotic susceptibility profile

The antibiotic susceptibility of *E. coli* strains was done as per CLSI guidelines version 2016. The antibiotic discs (Roscoe-Denmark) used were as follows: amoxicillin (10µg), fosfomycin (200µg), cefoxitin (30µg), ceftazidime (30µg), cefotaxime (30µg), cefepime (30µg), gentamicin (10µg), tetracycline (30µg), trimethoprim-sulfamethoxazole (25µg), imipenem (10µg), meropenem (10µg), ciprofloxacin (5µg), chloramphenicol (30 µg), piperacillin- tazobactam (10/100µg), and ciprofloxacin (5 µg) (concentration per disk). The *E. coli* ATCC 25922 and *Staphylococcus aureus* ATCC25923 standard strains were used as the quality control for the susceptibility results.

3.3. Carba NP-test

The Carba NP-test was performed for the phenotypic determination of carbapenemase enzymes; especially MBLs. As per CLSI guideline version 2016, a solution consisting of H2O, MgSO4, phenol red, and 6 $mg.mL^{-1}$ imipenem was prepared, of which 100µL was distributed in each cryo-tube. A loop of each isolate overnight growth was added to each tube and incubated for 1 to 2 hours. An alteration from red to yellow color showed imipenem hydrolysis and positive result.

3.4. Imipenem MIC determination

The MIC of imipenem was performed with the agar dilution method. The range of imipenem dilutions added to the Mueller Hinton agar was from 0.25 to 64 $\mu g.mL^{-1}$.

3.5. Detection of antibiotic resistance genes and virulence factors

Specific primers and PCR conditions used for the detection of genes encoding resistance enzymes and virulence factors among isolates are exhibited in Table 1. For all the reactions, the PCR thermal cycler device (BioRad- USA) was employed. The gel electrophoresis containing 1.5% agarose, a 100bp DNA marker, and safe stain (Sinagen, Iran) was used. The annealing temperature for each PCR condition is depicted in Table1.

3.6. Data analysis

Data of this study were analyzed using Graphpad prism 6.1 software and Chi-square test and ANOVA test, and results were considered as significant with a p value <.05 and 95% confidence interval.

4. Results

From a total of 50 imipenem-resistant *E. coli* isolates isolated from three hospitals, all were susceptible to fosfomycin, and the majority (86%) of them were susceptible to amikacin. The antibiotic resistance rates were as follows: tetracycline 88%, amoxicillin 100%, sulfonamide 60%, trimethoprim 58%, cefoxitin 100%, ceftazidime 100%, cefotaxime 100%, cefepime 90%, gentamicin 22%, trimethoprim-sulfamethoxazole 34%, imipenem 100%, meropenem 100%, ciprofloxacin 32%, chloramphenicol 22%, and piperacillin- tazobactam 54%.

4.1. The imipenem MIC

The imipenem MIC results showed that 24 isolates had MIC=2, 3 isolates had MIC=4, 8 isolates had MIC=8, 3 isolates had MIC=16, and 2 isolates had MIC=32. According to the CLSI guidelines, 13 isolates were carbapenem-resistant *E. coli*.

The genes mediating resistance were as follows: beta-lactams OXA-48 (8%), KPC-2 (0%), IMP (16%), VIM (0%), NDM-1 (0%), *fosA3* (0%), quinolones *qnrA* (28%), sulfonamide *sul1* (48%), and colistin *mcr-1*(0%).

As shown in Table 2, 5 IMP positive *E. coli* isolates showing MICs ranging from 8 to 32 $\mu g.mL^{-1}$ were detected from 5 male and 3 female patients with the age ranges from 33 to 61 years, 3 of which were OXA-48 producers as well. Five of these isolates were isolated from urinary tract infections, two isolates from stool, and one isolate from wound infections. Most of these isolates were multiple drug-resistant *E. coli*.

Furthermore, the prevalence rate of *fimA*, *hlyA*, *cnf1*, *vat*, *pic*, *crl*,and *papH* were 88, 36, 28, 10, 12, 44, and 88%, respectively. The distribution of virulence genes among various clinical sites are displayed in Table 2. There was significant difference among infection sites regarding the prevalence rate of virulence genes (95% CI, p value<.05).

5. Discussion

The increase in drug resistance especially resistance to carbapenems among *E. coli* pathotypes has led to fatality and difficulty in infections treatment. In this study, all the isolates were susceptible to fosfomycin, and 86% were susceptible to amikacin. In a study by Pullukcu et al. (2007), the microbiological and clinical success of fosfomycin on the drug-resistant isolates was 78.5 (41/52) and 94.3% (49/52), respectively (18). In another study by Oteo et al. (2010), in 231 ESBL-producing *E. coli* isolates, the rate of fosfomycin resistance had increased 9.1% during the years associated with fosfomycin parallel consumption in the community (19). In this study, from a total of 50 carbapenem-resistant *E. coli* strains, 13 isolates showed imipenem MIC≥8, which place them in the resistance range, and all were positive for *fimA* and *papH* genes, indicating virulent and resistant strains. The prevalence rate of carbapenemase and other resistance genes was determined as follows: OXA-48, 8%; KPC-2, 0%; IMP, 16%; VIM, 0%; NDM-1, 0%; *fosA3*, 0% as beta-lactamases; *qnrA*, 48%; *sul1*, 48%; and colistin *mcr-1*, 0%. It was observed that 8 isolates were able to amplify the IMP gene as the predominant metallo-beta lactamase (MBL) gene. Similarly, in a study by Alizadeh et al. (2015) in Kerman, none of ESBL-producing *E. coli* isolates were positive for bla_{IMP} and bla_{VIM} genes (20).

In previous study by Nobari et al. (2014) conducted in Iran, 3 and 5 out of 180 *E. coli* isolates harbored NDM1 and VIM genes, respectively (21). Of 92 carbapenem-resistant *E. coli* isolates in Hong Kong, one ST131/bla_{IMP-4} was detected (22). The blaIMP subtypes have been reported worldwide in Europe and United States (23-25). Several of them have been related to special clonal complexes and phylo groups; however, in this study, the clonal complexes were not detected. In this study, none of the isolates were positive for bla_{NDM1}, bla_{VIM}, and *fosA3* genes. The blaNDM1 has an endemic state in India, but has spread to several other parts of the world such as Europe, East Asia, South America, United States and Iran (26-30).

E. coli strains are the predominant cause of urinary tract and intestinal and even extra-intestinal infections by producing a number of virulence factors facilitating the colonization and invasion of the strains to host cells (31-33). Our data exhibited that the prevalence rate of UTIs was higher in female than in male patients. In addition to a host of factors, other factors such as alterations in the normal

vaginal flora in women can be a highrisk factor for developing urinary tract infections. In previous study by Fattahi et al. (2015) conducted in Iran, among UPEC isolates, 94 (94%) cases were *fimA* positive, this gene was associated with biofilm formation by these isolates (34). In another study, it was shown that colicin and microcin toxins were significantly more common among *fimA*-possessing *E. coli* isolates (35). The *cnf* gene which is involved in the renal damage is present in one third of *E. coli* strains causing pyelonephritis (36). Moreover, *cnf1* is responsible for the phagocytosis of polymorpho-nucluer cells and the apoptosis of epithelial cells. The predominant virulence factor produced by UPEC strains is a lipoprotein called *hlyA* which is involved in ascending the urinary tract infections rate, like pyelonephritis (37). A study from Mexico showed that 62, 7.4, and 6.5% of UPEC strains were positive for *papC*, *hlyA*, and *cnf1* genes, respectively, which were lower than our results (38). The high prevalence rate of antibiotic resistance and virulence factors determinants among imipenem-resistant *E. coli* isolates emphasizes the risk of fatal infections with no response to the treatment. In India, the *vat* toxin was detected in 51% of septicemia and 12% of fecal samples (4) while the prevalence rate of *pic* gene was 9%, these results were higher and lower than the result of this study, respectively. Another study in Germany demonstrated that 10 (35.7%), 8 (28.6%), and 7 (25%) UPEC strains were positive for *vat*, *hly*, and *cnf1* genes, respectively (39). The previous study in Zabul, South East Iran, detected the *vat* toxin in 18% of *E. coli* isolates (40). The difference in the prevalence rate of toxin and adherence genes among different studies might be due to the epidemiological differences, strains, and clonal complexes. In this study, the prevalence rate of *papH* was 88%. The strains from UTI and stool were not significantly different regarding the prevalence of virulence factors while the difference was significant (p value<.05) compared to wound and blood isolates. It should be taken into account that in this study, the number of isolates was low; thus, for obtaining more valid results, more isolates are needed to be investigated.

Table 1. The specific primers used in this study.

Primer	Sequence 5' → 3'	Amplicon size	Annealing T (℃)	Reference
fosA3	F: GCGTCAAGCCTGGCATTT R: GCCGTCAGGGTCGAGAAA	282	56	(10)
KPC-2	F: TTGCCGGTCGTGTTTCCCTTTAGC R: GGCCGCCGTGCAATACAGTGATA	282	65	This study
OXA-48	F: CGCCCGCGTCGACGTTCAAGAT R: TCGGCCAGCAGCGGATAGGACAC	484	65	This study
IMP	F: GGGTGGGGCGTTGTTCCTA R: TCTATTCCGCCCGTGCTGTC	182	61	This study
VIM	F: CATTGTCCGTGATGGTGATGAGT R: GCGTGTCGACGGTGATGC	205	60	This study
NDM-1	F: CGCACCTCATGTTTGAATTCGCC R: GTCGCAAAGCCCAGCTTCGC	1015	63	(11)
qnrA	F: TCAGCAAGAGGATTTCTCA R: GGCAGCACTATTACTCCCA	627	60	(12)
mcr-1	F: CGCGACCGCCAATCTTACC R: CCCAATCGGCGCATCAAACC	396	61	This study
fimA	F: GCACCGCGATTGACAGC R: CGAAGGTTGCGCCATCCAG	132	61	This study
hlyA	F: GGT GCA GCA GAA AAA GTT GTAG R: TCT CGC CTG ATA GTG TTT GGT	1551	59	(13)
cnf1	F: GGGGGAAGTACAGAAGAATTA R: TTGCCGTCCACTCTCACCAGT	1112	60	(14)
vat	F: TCCTGGGACATAATGGCTAG R: GTGTCAGAACGGAATTGTC	930	61	(15)
pic	F: ACTGGATCTTAAGGCTCAGG R: TGGAATATCAGGGTGCCACT	410	58	(15)
crl	F: TTTCGATTGTCTGGCTGTATG R: CTTCAGATTCAGCGTCGTC	250	59	(16)
papH	F: TTAAAGATAATCGGGTCAT R: GGAATCAGAGAAAAGGTT	858	59	(17)

Table 2. The presence of virulence genes among five strains with high (>8µg.mL^{-1}) MIC against imipenem.

Isolate	Infection site	MIC_{IMI} µg.ml^{-1}	*fimA*	*hlyA*	*cnf1*	*vat*	*pic*	*crl*	*papH*
1	Stool	32	+	+	+		+	+	+
2	Stool	32	+			+		+	+
3	UTI	32	+	+					+
4	UTI	32	+	+	+	+	+	+	+
5	UTI	32	+						+

6. Conclusion

In this study, all imipenem-resistant *E. coli* isolates were susceptible to fosfomycin, and all were negative for *fosA3* gene. Among carbapenemase genes, IMP and OXA-48 type enzymes were detected, which were associated with higher MIC levels (8 to 32µg.mL^{-1}). In this study, data suggest the role of these carbapenemases in resistance to carbapenems. Furthermore, the presence of multiple drug resistance (high level MICs) strains encoding adhesive and secretory virulence factors is a concern for the infections treatment.

Conflict of interest

The authors note no conflict of interest.

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Authors' Contributions

The study was designed and perforemed by Farshad Nojoomi and Abdolmajid Ghasemian.

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