

Detection of *qacEΔ1*, *qacG*, *qacE*, *qacF* resistance genes in *Escherichia coli* producing broad-spectrum beta-lactamases to benzalkonium chloride

F. Hadadi (MSc)¹, E. Ghaznavi Rad (PhD)², A. Almasi-Hashiani (PhD)³, H. Abtahi (PhD)^{*2}

1.Students Research Committee, Arak University of Medical Sciences, Arak, I.R.Iran

2.Molecular and Medicine Research Center, Arak University of Medical Sciences, Arak, I.R.Iran

3.Traditional and Complementary Medicine Research Center, Arak University of Medical Sciences, Arak, I.R.Iran

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ABSTRACT

BACKGROUND AND OBJECTIVE: The resistance genes of quaternary ammonium compounds(*qac*) play an important role in the resistance of gram-negative bacteria producing broad-spectrum beta-lactamases to disinfectants. The aim of this study was detection of *qacEΔ1*, *qacG*, *qacE*, *qacF* resistance genes in *Escherichia coli* producing broad-spectrum beta-lactamases to benzalkonium chloride.

METHODS: This study cross sectional-descriptive was conducted on 150 clinical samples of selected hospitals in Arak. ESBL strains were identified by using phenotypic methods of disc diffusion and combinatory disc method and evaluating the SHV, TEM, CTXM1 genes by genotyping method. The PCR was performed to determine the resistance genes *qacEΔ1*, *qacG*, *qacE* and *qacF*.The electrophoresis of PCR products and the MIC of benzalkonium chloride were relative to *E. coli* producing ESBL. Antibiotic pattern of *Escherichia coli* (ESBL), quadruple ammonium resistance genes and benzalkonium chloride MIC were also investigated.

FINDINGS: This study showed that 60% of *Escherichia coli* were ESBL producer. The *qacEΔ1* genes were observed in all of them and *qacE*, *qacF*, *qacG* genes were not found in any of the strains. The strains had MIC range from 32 to 64 mg/l for benzalkonium chloride. Resistance to carbapenems (33.33%) was observed.

CONCLUSION: This study showed that *qacEΔ1* resistance gene and resistance to disinfectant benzalkonium chloride increased. Also increased resistance to the antibiotics studied were observed in *E. coli* ESBL strains.

KEY WORDS: *Escherichia Coli*, *Beta-Lactamase*, *Benzalkonium Chloride*, *Disinfectant*.

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*Corresponding Author: H. Abtahi (PhD)

Address: Department of Microbiology, University of Medical Sciences, Basij Sq., Arak, I.R.Iran

Tel: +98 86 34173530

E-mail: abtahi@arakmu.ac.ir

Introduction

Beta-lactamases are enzymes that by hydrolysis of the beta-lactam ring inactivate these antibiotics and thereby cause resistance to the beta-lactam antibiotics. A new class of these enzymes, called broad-spectrum beta-lactamases, encodes genes that are transposable on large conjugated plasmids and can simultaneously transfer resistance genes to other antimicrobial agents, including bacterial resistance to antibiotics (1-4). Numerous reports from different regions of the country show the prevalence of broad-spectrum beta-lactamase-producing organisms from 40 to 80% among gram-negative bacteria (5-7), which has been of concern to physicians.

Infection with these bacteria has been associated with higher mortality rates, increased hospital stay, and increased treatment costs (8). *Escherichia coli* is one of the common pathogens of nosocomial infections that has become resistant to beta-lactam antibiotics including cephalosporins due to the acquisition of plasmids encoding broad-spectrum beta-lactamases. Three genes of beta-lactamases including SHV, CTX^{M1} and TEM are mostly found in *Escherichia coli* (9).

In recent years, significant efforts have been made to promote health and infection control in hospitals and in the community, leading to increased use of disinfectants. For example, disinfectants based on quaternary ammonium compounds such as benzalkonium chloride are widely used in hospitals to disinfect surfaces and prevent infections because these compounds have broad antimicrobial activity (10,11). In gram-negative bacteria, *qacE*, *qacEΔ1*, *sugE* (P), *qacF* and *qacG* genes encode resistance to quaternary ammonium compounds (QACs genes) that are located on removable genetic elements (12-14).

As a result of the deletion mutation on *qacE*, the *qacEΔ1* gene is formed that encodes resistance to disinfectants such as quaternary ammonium compounds. The *qacF* gene is genetically similar (68%) to the *qacE* gene (15). In gram-negative bacteria, *qacE* and *qacEΔ1* genes are much more prevalent than other QACs genes (16,17). Among ESBL isolates isolated from clinical specimens, there was a significant relationship between the presence of *qacE* and *qacEΔ1* resistance genes with antibiotic resistance (18). *Escherichia coli* isolated from clinical sources also showed a high MIC compared to quaternary ammonium compounds, which is associated with antibiotic resistance (19). Few studies have been performed on the abundance of quaternary ammonium resistance genes in

ESBL *Escherichia coli*. Since the control of bacterial diseases depends on the use of antibiotics, the spread of antibiotic resistance phenomenon is raising public concern and is one of the public health problems. The present study was performed to investigate the antibiotic pattern, the prevalence of ESBL *Escherichia coli*, the distribution of *qacE*, *qacG*, *qacF* and *qacEΔ1* resistance genes in this bacterium and to determine the minimum inhibitory concentration of benzalkonium chloride compared to ESBL *Escherichia coli* isolated from clinical specimens.

Methods

Samples: This cross-sectional study was approved by the Ethics Committee of Arak University of Medical Sciences under code ARAK.MU.REC.IR.294.1396 and was performed on 150 clinical samples (urine, blood, sputum and ulcer) in 1397 patients referred to selected training hospitals in Arak. Samples collected from hospitals were transferred to the University Microbiology Laboratory by the Stuart Transition Environment. Samples were cultured on blood agar and Meccanaki medium (Merck, Germany) for bacterial identification. *Escherichia coli* were identified using biochemical methods such as gram staining, growth on VP, MR, SIM, TSI, OF, Simon citrate, urea, lysine decarboxylase (20).

Determination of susceptibility pattern of *Escherichia coli* isolates by Disk diffusion agar: *Escherichia coli* strains were investigated on the Müller Hinton Agar medium (Merck, Germany) in terms of antibiotic susceptibility pattern by disk diffusion method (Kirby-Bauer method) and based on Clinical Laboratory Standards Institute guidelines (2018 CLSI). Antibiotics (Mast, UK) included: Nitrofurantoin (300 µg), Tri-Metoprim Sulfamethoxazole (5 µg), Gentamicin (10 µg), Ciprofloxacin (5 µg), cefepime (30 µg), nalidixic acid (30 µg), amikacin (30 µg), cefotaxime (30 µg), cefazolin (30 µg), cefaxime (5 µg), imipenem (10 µg), meropenem (10 µg), Tetracycline (30 µg), ceftazidime (30 µg), ceftazidime (30 µg), ceftazidime (30 µg). E-coli 25922 was also used as control strain. After disk diffusion, plates were incubated for 24 h at 37 °C, then the diameter of the growth zone around each disk was interpreted based on the 2018 CLSI tables (21).

Phenotypic confirmation of ESBL producing isolates: Combination disk method was used to confirm the phenotypic characterization of ESBL isolates. In the above method, discs (Mast, UK) of ceftazidime (30 µg),

ceftazidime-clavonic acid (10-30 µg), cefotaxime (30 µg) and cefotaxime-clavonic acid (10-30 µg) were used. The suspension was made from a bacterium equivalent to Half-McFarland and cultured on Müller Hinton agar medium (Merck, Germany). The discs were then placed on the perimeter. After 24 h incubation at 37 °C, the diameter of the growth zone around each disk was measured. As the diameter of the bacterial growth zone around the compound disc was 5 mm higher than the single antibiotic growth zone around the single disk, the bacterium had broad-spectrum beta-lactamase enzymes (ESBLs), and the result was a positive bacterial phenotypic test. The standard strain of *Escherichia coli* ATCC 35218 was used to control the quality of ESBL isolates (21). Then ESBL *Escherichia coli* isolates were stored in Luria Brittany Broth (LBB) medium containing 20% glycerol at -20 °C.

Genotypic identification of ESBL strains: Identification of SHV, TEM and CTXM1 beta-lactamase genes was performed by PCR using specific primers prepared (Table 1) (22).

Identification of *qacE*, *qacEA1*, *qacF* and *qacG* genes by polymerase chain reaction (PCR): After preparation of a fresh culture from stored ESBL-producing *Escherichia coli* isolates, DNA extraction using kit (Experiment Kit, Iran) was done. The sequences of primers for construction was delivered to Gene Fanavaran Co. (TAG Copenhagen's sole agent in

Iran). Then, the identification of *qacE*, *qacEA1*, *qacF* and *qacG* genes in ESBL *Escherichia coli* isolates was performed with the help of specific primers prepared (Table 2) (23).

PCR products electrophoresis: The PCR product obtained for each gene was identified by electrophoresis on 1% agarose gel. The bands were then observed and photographed using a UV transducer and gel dock (Fig 1).

Determination of Minimum Inhibitory Concentration: Minimum inhibitory concentration of ESBL *Escherichia coli* isolates relative to benzalkonium chloride 4% was determined by microdilution broth in accordance with CLSI 2018 guidelines. In this method, serial dilutions of the disinfectant were prepared. The suspension was made from a bacterium equivalent to half McFarland. Finally, bacterial concentration was 5 *10⁵ in each well. The microplate was then incubated at 37 °C for 24 hours. Serial dilutions ranged from 1/2 to 1/1024 and the concentration range for MIC determination was 0.125 to 1024 mg/L. *E. coli* ATCC 25922 was used as quality control (21). Antibiotic pattern, prevalence of ESBL *Escherichia coli*, distribution of *qacE*, *qacG*, *qacF* and *qacEA1* resistance genes in this bacterium and minimum inhibitory concentration of benzalkonium chloride were evaluated and STATA 13 software was used to calculate the number and percentage.

Table 1. Characteristics of the beta-lactamase gene primers used in PCR

Primer	Sequence(5 to 3)	Amplicon Size(bp)	Annealing Temperature
TEM	ATGAGTATTCAACATTTCGG CTGACAGTTACCAATGCTTA	867	58
SHV	GATGAACGCTTTCCCATGATG CGCTGTTATCGCTCATGGTAA	214	61
CTXM1	AAGACTGGGTGTGGCATTGA AGGCTGGGTGAAGTAAGTGA	670	52

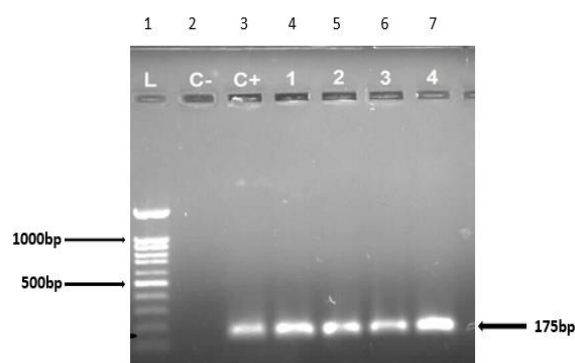


Figure 1. PCR results of the *qacEA1* gene in ESBL strains of *Escherichia coli*. Column 1: 100 bp size marker, column 2: negative control strain (without *qacEA1* gene), column 3: positive control strain (with *qacEA1* gene), Column 4 to 7: Genotype-positive strains with *qacEA1* resistance gene

Table 2. The qac primers used in this study

Gene	Sequence (5'-3')	Annealing temperature (elongation time)	PCR product size (bp)	Accession no.
qacEΔ1	AATCCATCCCTGTCCGGTGTT	56°C (30s)	175	JN596280
	CGCAGCGACTTCCACGATGGGGAT			JN566044
qacE	AAGTAATCGCAACATCCG	50°C (30s)	258	X68232
	CTACTACACCACTAACTATGAG			HQ875011
qacF/H/I	GTCGTCGCAACTTCCGCACTG	60°C (30s)	229	FJ160769
	TGCCAACGAACGCCACACA			JN596279
qacG	TGCCAACGAACGCCACACA	56°C (30s)	122	FJ950725
	AACGCCGCTGATAATGAA			AF288045

Results

Samples included 123 urines (82%), 11 blood (7.3%), 11 ulcers (7.3%) and 5 sputa (3.4%). Of the 150 samples studied, 130 *Escherichia coli* were isolated. 78 isolates (60%) of the 130 *E. coli* isolates were ESBL positive. The results of genotypic testing revealed that all ESBL positive *Escherichia coli* had CTXM1 gene. 45 isolates (57.69%) had SHV gene and 55 (70.51%) isolates had TEM gene. According to the antibiotic susceptibility pattern of ESBL *Escherichia coli*, there was the least resistant to nitrofurantoin and resistance to most antibiotics was increased, including carbapenem, which was 33.33% (Fig 2).

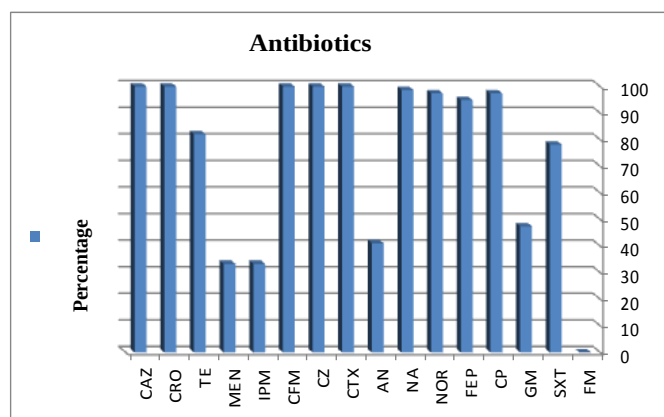


Figure 2. Frequency distribution of resistance pattern of *Escherichia coli*-ESBL isolates to antibiotics studied

FM (Nitrofurantoin), SXT (trimethoprim sulfomethoxazole), GM (gentamicin), CP (ciprofloxacin), FEP (cefepime), NOR (neurotomicin), NA (nalidixic acid), AN (amikacin), CTX (cefotaxime) (Cefazolin), CFM (cefexime), IPM (imipenem), MEN (meropenem), TE (tetracycline), CRO (ceftriaxone), CAZ (ceftazidime).

The sensitivity results of the benzalkonium chloride disinfectant showed that the MIC in ESBL strains was between 32-64 mg/l that 43.64% of the samples had 64

mg/l concentration and 56.36% of samples had 32 mg/l concentration. The qacEΔ1 gene was found in all ESBL *E. coli* isolates, and none of the qacG, qacE, qacF genes were detected in any of the isolates.

Discussion

In this study, the qacEΔ1 gene was detected in all isolates (100%). The qacE, qacEΔ1, qacF, qacG resistance genes are encoded on the plasmid or integron that can induce resistance to QACs by the efflux pump (12,13,15). ESBL *Escherichia coli* is able to withstand a greater amount of disinfectants by expressing the resistance gene such as the qacEΔ1 gene to quaternary ammonium compounds.

Therefore, excessive use of disinfectant not only does not eliminate or eliminate the bacteria but can also lead to resistance of ESBL bacteria to disinfectants. In the study of Canal et al., the qacEΔ1 gene was detected in all isolates (100%) (24). Shafaati et al. reported the abundance of the qacEΔ1 gene among ESBL-producing *Escherichia coli* isolates from urine samples about 94% (18). Mahzonieh et al. reported 91.5% of qacEΔ1 resistance genes in gram-negative bacteria (25). In this study, all ESBL strains had minimum inhibitory concentrations ranging from 32 to 64 mg/L for benzalkonium chloride. In the study of Guo et al., the minimum inhibitory concentration of ESBL strains was reported to be between 8 to 128 mg/L (23).

In the study of Liu et al., the minimum inhibitory concentration of benzalkonium chloride for *S. baumannii* and *Klebsiella* was 8.32 mg/l and 128-32, respectively (26). Given the increased inhibitory concentration of quaternary ammonium compounds of benzalkonium chloride in this study, there is more need to pay attention to the choice of disinfectant and further research on its use and also is necessary the proper use of antiseptics and disinfectants in hospitals for the

effectiveness of disinfectants. The present study showed that the most effective antibiotic for ESBL *Escherichia coli* is nitrofurantoin, which was consistent with the study by Amiri et al. in 2015 and Ranjbaran et al. (5, 27). Resistance to carbapenems has also increased compared to previous studies (7,26,28), indicating an increase in carbapenem use in hospitals. It should be noted that carbapenems are often the preferred option for the treatment of ESBL-producing bacterial infections as well as the treatment of severe infections acquired from the hospital. Therefore, unnecessary use, and irrational administration of antibiotics, can lead to drug resistance.

Therefore, the high prevalence of ESBL-producing *Escherichia coli* and its increased qac resistance genes, as well as resistance to broad-spectrum antibacterial agents (carbapenems) in Enterobacteriaceae, are of concern in this study. There is a need for infection control measures to rationally manage antibiotic use and rapid identification of beta-lactamase producing strains. In order to prevent the increase of drug resistance in different regions, it is recommended to conduct continuous research in each area. Because rational administration of antibiotics helps the effectiveness of the treatment process. On the other hand, increasing the

minimum inhibitory concentration in this study indicates that proper disinfectants should be used in hospitals in order to be effective disinfectants. Use of disinfectants at concentrations lower than the inhibitory concentration may increase the prevalence of bacteria resistant to disinfectants, which may spread the infection to patients and the community. Despite the prevalence of QAC resistance genes in *Escherichia coli*, the mechanism of resistance to disinfectants and the relationship between resistance genes and antibiotic susceptibility in *Escherichia coli* isolates have not been well understood. Therefore, increasing the recognition of the resistance genes of quaternary ammonium compounds in ESBL *Escherichia coli*, proper use of disinfectants, as well as investigating the microbial resistance patterns in this bacterium, may help the effectiveness of infection control process in hospitals.

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