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Integron-Mediated Multidrug and Quinolone Resistance in Extended-Spectrum β-Lactamase-Producing *Escherichia coli* and *Klebsiella pneumoniae*

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Abstract

Background: Despite intensive care and treatment strategies, the development of antibiotic resistance to empirical drugs is concerning.
Objectives: The aim of this study was to characterize extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli* and *Klebsiella pneumoniae* for integron-mediated quinolone resistance and multidrug resistance (MDR).
Methods: In this cross-sectional study, 71 *E. coli* and 63 *K. pneumoniae* clinical isolates underwent antibiotic susceptibility testing with the Kirby-Bauer method, followed by ESBL phenotypic screening with the combination disc method. The isolates were then genotypically characterized with PCR for the presence of integrons and the *gyrA*, *parC*, $bla_{\text{CTX-M-3}}$, bla_{TEM}, and bla_{SHV} genes. Resistance to antibiotics was confirmed by sequencing.
Results: *K. pneumoniae* was a potent ESBL producer (71.4%) in comparison to *E. coli* (57.7%). The predominant ESBL genotypes in *E. coli* and *K. pneumoniae* confirmed by sequencing were $bla_{\text{CTX-M-15}}$ (67.60%) and $bla_{\text{SHV-1}}$ (80.95%), respectively. Imipenem was the only antibiotic active against the ESBL-producing isolates. Approximately 54% of the isolates exhibited MDR patterns. MDR was more frequently related to the presence of $bla_{\text{CTX-M-3}}$ in comparison to other genotypes. The prevalence of class 1 integrons was 15 (45.4%) and 22 (66.6%) of the *E. coli* and *K. pneumonia* isolates, respectively. Within the ESBL group, a class 1 genetic element was associated with the $bla_{\text{CTX-M-3}}$ genotype in *E. coli* (36.58%) and *K. pneumoniae* (51.11%). Overall, almost half of the ESBL producers, irrespective of genus, were simultaneously resistant to quinolones. The simultaneous presence of class 1 and 2 integrons in quinolone-resistant isolates was the most frequent observation.
Conclusions: The high prevalence of multidrug and ESBL-mediated resistance is a therapeutic concern. The co-emergence of ESBLs and quinolone resistance in *E. coli* and *K. pneumoniae* suggests the preservation of the power of antibiotics in the face of the antibiotic-resistance crisis.

Keywords: Drug Resistance, Extended-Spectrum β-Lactamase, Quinolones, *Klebsiella pneumoniae*, *Escherichia coli*

1. Background

Despite increased care and treatment strategies, infections in burn patients remain a concern due to morbidity and mortality (1). Immunodeficiency, burn-wound infections, and inappropriate therapy can lead to microbial invasions (2). *Escherichia coli* and *Klebsiella pneumoniae*, normal flora of the gastrointestinal tract, mimic other pathogens and either colonize sterile sites or act as opportunistic pathogens (3, 4), when they find a better niche. Infections caused by these bacteria can be successfully treated with antibiotics, such as quinolones and β-lactams; however, a high prevalence of quinolone resistance in *Enterobacteriaceae* has been described (5). In *E. coli*, quinolone resistance is frequently found among strains producing extended-spectrum β-lactamase (ESBL) compared to ESBL-negative strains (6-8). A major concern within hospitals is the spread of ESBL-positive bacteria, which may lead to outbreaks or endemic occurrences. Thus, it is necessary to investigate the prevalence of ESBL-positive strains in hospitals in order to formulate a policy of empirical therapy in high-risk units where infections due to resistant organisms are much more common. The fact that ESBL genes could be acquired by strains harboring particular integrons may enlarge the possibilities for selecting for these isolates with a variety of different antimi-

crobials. ESBLs feature bla_{TEM}, bla_{SHV}, and bla_{CTX-M} genes (9, 10). Five integron classes related to antibiotic resistance have been described based on the homology of their integrase genes; among the *Enterobacteriaceae*, class 1 integrons have been correlated with high antimicrobial resistance (11, 12).

2. Objectives

The present study was conducted to report integron-mediated multidrug resistance (MDR) and quinolone resistance in ESBL-producing *E. coli* and *K. pneumoniae* isolates.

3. Methods

Bacterial isolates and antibiotic susceptibility testing

This cross-sectional study was conducted on routine and non-duplicate clinical specimens, including samples from blood, wounds, urine, endotracheal tubes, and various bodily fluids. The specimens were sent from burn wards and intensive care units (ICUs) to the Division of Microbiology, Sina hospital of Tabriz, from June to December 2014. The samples were processed for the isolation and identification of *E. coli* and *K. pneumoniae* according to phenotypic methods as described elsewhere (13, 14). Wound and urine specimens yielding $> 10^5$ colony-forming units (CFUs/mL) were included in this study. Duplicate isolates from the same patient were not included.

Antibiotic susceptibility testing was performed on all isolates using the Kirby-Bauer method as described by the clinical and laboratory standards institute (CLSI) (15) with a panel of the following β-lactam antibiotic discs: cefazolin (CZ) (30 μg), ceftazidime (CAZ) (30 μg), ceftriaxone (CTR) (30 μg), cefepime (CFM) (30 μg), cefotaxime (CTX) (30 μg), and imipenem (IMI) (10 μg). The following non-β-lactam antibiotics were also tested: nalidixic acid (NA) (30 μg), ciprofloxacin (CP) (5 μg), amikacin (AK) (30 μg), gentamicin (GA) (10 μg), trimethoprim-sulfamethoxazole (cotrimoxazole, COT) (1.25/23.75 μg), and ofloxacin (OFL) (10 μg) (Mast Diagnostics, Merseyside, UK).

3.1. Detection of Quinolone Resistance

Quinolone resistance was detected phenotypically with the disc diffusion test described above and confirmed by ciprofloxacin and nalidixic acid E-tests (Liofilchem, Italy). The criterion for ciprofloxacin resistance was a minimal inhibitory concentration (MIC) of $\geq$ 4 μg/mL, and an MIC of $>$ 32 μg/mL for nalidixic acid, according to the CLSI breakpoint criteria (15). *E. coli* ATCC 25922 used as a positive control for both the MIC and the disc diffusion tests.

3.2. Detection of ESBL Production

The ESBL phenotypic detection test was performed with the combination disc method, using ceftazidime, cefpodoxime, cefotaxime, aztreonam (30 μg), and ceftriaxone (30 μg) discs (Mast Diagnostics, Merseyside, UK), with the inhibitory effect of clavulanic acid as recommended by the CLSI (15). *E. coli* ATCC 25922 was used as the ESBL-negative control strain and *K. pneumoniae* ATCC 700603 was used as the ESBL-positive control strain. ESBL production was confirmed using ceftazidime/ceftazidime plus clavulanic acid (CAZ/CAZCV) ESBL strips (Liofilchem, Italy) according to the manufacturer's instructions, as CV-clavulanic acid is a β-lactam inhibitor. The results were analyzed based on the decrease in MIC of ceftazidime in the presence of the inhibitor (15).

3.3. Detection of β-Lactamases by PCR

To obtain template DNA, a single colony from each isolate was inoculated overnight in Luria–Bertani broth (16), and the genomic DNA of each isolate was extracted using a Bacterial DNA Kit (Cina Gene Co.). Detection of the *bla* genes was performed with polymerase chain reaction (PCR) using a panel of specific primers for bla_{SHV}, bla_{TEM}, and $bla_{CTX-M-3}$, as described previously (17-19). The primers for TEM were (forward) 5'-AGATCAGTTGGGTGCACGAG-3' (nucleotides (nt) 313 - 332) and (reverse) 5'-TGCTTAATCAGTGAGGCACC-3' (nt 1061 - 1042). The primers for SHV were (forward) 5'-GGGAAACGGAACTGAATGAG-3' (nt 606 - 625) and (reverse) 5'-TTAGCGTTGCCAGTGCTCG-3' (nt 988 - 970). PCR amplification of the allele belonging in the $bla_{CTX-M-3/15/22}$ group was carried out with primers CTX-M3G-F (5'-GTTACAATGTGTGAGAAGCAG) and CTX-M3G-R (5'-CCGTTTCCGCTATTACAAAC).

The amplification was carried out as uniplex PCR, then cycling conditions were standardized for multiplex PCR as follows: initial denaturation at 94°C for 7 minutes; denaturation at 94°C for 50 seconds, annealing at 50°C for 40 seconds, and elongation at 72°C for 60 seconds, repeated for 35 cycles; and a final extension at 72°C for 5 minutes. PCR was carried out in 25 μL volumes with 30 pmol of each primer, 200 mM of deoxynucleoside triphosphate, 1.5 mM of $MgCl_2$, and 0.5 U of Taq polymerase. PCR products were electrophoresed on 2% gel and analyzed with the UV Gel Documentation System. PCR products were purified using the PCR Preps DNA Purification System (Bioneer, Korea). All bidirectional nucleotide sequencing was performed using the abovementioned PCR primers.

3.4. Detection of Quinolone Resistance by PCR

The *gyrA* gene was amplified with primers 5'-TTAATGATTGCCGCCGTCGG-3' and 5'-

TACACCGGTCAACATTGAGG-3' (yielding a 648-bp product) and *parC* was amplified with primers 5'-AAACCTGTTCAGCGCCGCATT-3' and 5'-GTGGTGCCGTTAAGCAAA-3' (yielding a 395-bp product) to amplify the quinolone-resistance-determining region (QRDR) present in all clinical isolates (20, 21).

PCR for *gyrA* was carried out in volumes of 50 μL containing 2.5 U of Taq polymerase along with standard PCR buffer (10 mM of Tris-HCl, pH 8.3, 50 mM of KCl, 1.5 mM of $MgCl_2$), 200 mM of each dNTP (Ready Master Mix, Pishgam Biotech Co., Iran), and 10 pmol of each primer. The reaction was performed in a thermal cycler programmed for denaturation at 95°C for 7 minutes, and 30 cycles as follows: denaturation at 94°C for 30 seconds, annealing at 65°C for 30 seconds and elongation at 72°C for 2 minutes. The final extension was allowed at 72°C for 5 minutes (21). The amplification for *parC* was carried out as above but with a slight modification in the PCR reaction: initial denaturation at 93°C for 5 minutes, denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and elongation at 72°C for 1 minutes, repeated for 35 cycles, followed by a final extension at 72°C for 10 minutes (21). Ten microliters of product was electrophoresed on 2% agarose gel and analyzed with the UV Gel Documentation System. The corresponding specific PCR products were sequenced by Bioneer, Korea.

3.5. Detection of Integrase Genes

The multiplex-PCR method was used to detect class 1, 2, and 3 integrons (yielding PCR products of 475 bp, 789 bp, and 922 bp, respectively). The primers and the PCR protocol utilized in this study were as described previously (22).

3.6. Statistical Analysis

Categorical variables were compared by means of either χ^2 analysis or Fisher's exact test when needed. A 2-tailed P value of < 0.05 was considered significant. All statistical calculations were done using the standard programs in the statistical package for social sciences (SPSS) version 16.

4. Results

4.1. Bacterial Isolates and Antibiotic Susceptibility Testing

The present study was carried out on 71 (52.6%) *E. coli* and 63 (46.7%) *K. pneumoniae* isolates from various clinical specimens. The majority of *E. coli* isolates (n = 50) (70.4%) (compared to 16 *K. pneumoniae* isolates) were the etiologic agents of urinary tract infections in burn patients, while 26 (41.26%) of *K. pneumoniae* isolates (compared to nine *E. coli* isolates) were the core microbial agents of wound infections, a statistically significant association ($P < 0.001$). The other sources of these isolates were blood (12 *E. coli* and 19 *K. pneumoniae*) and endotracheal secretions (n = 2; only *K. pneumoniae*). No significant difference was observed in the prevalence of these two pathogens isolated from other clinical specimens.

The patterns of resistance to β-lactam and non-β-lactam antimicrobial agents among these isolates are shown in Table 1. Among the β-lactams tested, none of the cephalosporins were found to be effective for both of the organisms. Using the disc diffusion method, the results showed that 88.8% of *K. pneumoniae* isolates were not susceptible to ceftazidime, compared to 57.7% of the *E. coli* isolates. This had a two-tailed P value of < 0.0001 so the association was considered to be extremely statistically significant. Similarly, the majority of *K. pneumoniae* isolates were significantly more resistant ($P < 0.001$) to ciprofloxacin and ofloxacin as compared to the *E. coli* isolates. For the other β-lactam antimicrobial agents, *E. coli* and *K. pneumoniae* did not differ in susceptibility.

Interestingly, when the *E. coli* and *K. pneumoniae* isolates were tested for ESBL production with the disc diffusion method, *K. pneumoniae* was found to be a potent ESBL producer (n = 45; 71.4%) in comparison to *E. coli* (n = 41; 57.7%). However, this association was not statistically significant (P = 0.108). Ceftazidime was observed to be the most suitable substitute, over cefpodoxime and cefotaxime alone or in combination with clavulanic acid ($P < 0.05$). Imipenem was the only antibiotic observed to be active against ESBL-producing *E. coli* (n = 30; 73.17%) and *K. pneumoniae* (n = 41; 91.11%); however, the association between the groups and the outcomes was not quite statistically significant.

The results of the ESBL-encoding gene detection by PCR revealed that the *E. coli* and *K. pneumoniae* isolates harbored the bla_{TEM}, bla_{SHV}, and $bla_{CTX\text{-}M\text{-}3}$ β-lactamase genes at different frequencies. The predominant ESBL *E. coli* genotypes were $bla_{CTX\text{-}M\text{-}3}$ (n = 48; 67.60%) followed by bla_{TEM} (n = 33; 46.47%) and bla_{SHV} (n = 14; 19.71%), while in *K. pneumoniae*, bla_{SHV} was predominant (n = 51; 80.95%). The two-tailed P value was < 0.0001, which is considered to be extremely statistically significant. Both the bla_{CTX} and the bla_{TEM} genes were found in 37 (58.73%) of the *K. pneumoniae* isolates. Since no differences were found between the resistance patterns expressed by the *E. coli* and *K. pneumoniae* isolates, they were considered together. It is remarkable that three β-lactamase genes were also co-expressed in both ESBL-producing *E. coli* and in *K. pneumoniae*. The most common combination pattern was $bla_{TEM} + {}_{SHV} + {}_{CTX}$, bla_{CTX} with bla_{TEM} in 17 isolates, respectively, while $bla_{CTX} + {}_{SHV}$ was observed in 14 isolates, five of which concealed the presence of $bla_{SHV} + {}_{TEM}$ (Table 2).

The most frequent phenotype pattern of MDR was

Table 1. Antibiotic Susceptibility Patterns of *E. coli* and *K. pneumoniae*

Antimicrobial Agent	*E. coli* (n = 71)		*K. pneumoniae* (n = 63)		P Value
	Susceptible (%)	Non-Susceptible (%)	Susceptible (%)	Non-Susceptible (%)	
Ceftazidime	30 (42.25)	41 (57.74)	7 (11.11)	56 (88.8)	< 0.001
Cefepime	2 (29.57)	69 (47.18)	-	63 (100)	NS
Ceftriaxone	8 (11.26)	63 (88.73)	5 (7.93)	58 (92.06)	NS
Cefazolin	6 (8.45)	65 (91.54)	3 (4.76)	60 (95.2)	0.61
Cefotaxime	17 (7.04)	54 (92.95)	5 (15.87)	58 (84.12)	< 0.01
Ciprofloxacin	15 (21.12)	56 (78.87)	2 (3.17)	61 (96.8)	< 0.001
Ofloxacin	27 (30.02)	44 (61.97)	10 (15.87)	53 (84.12)	< 0.001
Nalidixic acid[a]	0	50 (100)	0	16 (25.3)	NS
Amikacin	57 (80.28)	14 (19.71)	51 (80.95)	12 (19.04)	NS
Gentamicin	28 (39.43)	43 (60.56)	25 (39.68)	38 (60.3)	NS
Imipenem	59 (83.09)	12 (16.9)	59 (93.65)	4 (6.34)	NS
Cotrimoxazole	30 (42.25)	41 (57.74)	25 (39.68)	38 (60.31)	NS

Abbreviation: NS, not significant.
[a] Nalidixic acid tested only for urine specimen positive for: *E. coli* (n = 50) and *K. pneumoniae* (n = 16).

CAZ-CFM-CTR-CZ-GA-COT, which was found in 86 (54.65%) isolates of ESBL-producing *E. coli* and *K. pneumoniae*, followed by CAZ-CFM-CTR-CZ-AK-GA-COT and CAZ-CFM-CTR-CZ, which were each observed in 29 (33.72%) isolates. The phenotypic resistance pattern CAZ-CFM-CTR-CZ-GA was identified in 19 (22.09%) of the isolates, while the CFM-CTR-CZ-AK-GA-IMI-COT phenotype was identified in 18 (20.93%). Interestingly, resistance to gentamicin, amikacin, imipenem, co-trimoxazole, and ceftazidime was more frequently correlated with the presence of bla_{CTX} in comparison to other genotypes, although the association was not significant. In *K. pneumoniae*, the panel of antibiotic resistance was associated with ESBL genotypes (Table 3). Further DNA sequence analysis of the bla_{TEM} sequences indicated that these two bacteria harbored the TEM-1b subgroup, whereas the subgroup found in the bla_{SHV} isolates was SHV-1. In the case of the $bla_{CTX\text{-}M\text{-}3}$ family, which consists of CTX-M-5, -15, and -22, these isolates were found to be carriers of the subgroup CTX-M-15 on sequencing.

4.2. Quinolone Resistance

In order to confirm the quinolone resistance of the isolates by disc diffusion, the MICs of ciprofloxacin and nalidixic acid were determined. The MICs of nalidixic acid ranged from 8 to > 256 μg/mL, and for ciprofloxacin, from 0.032 to > 32 μg/mL. The MIC50 and MIC90 values for nalidixic acid for all *E. coli* and *K. pneumoniae* isolates were found at the resistance breakpoints (both = 163.55 mg/L), whereas for ciprofloxacin, MIC50 and MIC90 was 24.78 mg/L.

4.3. Integrase Genes

Class 1 integrons were found more frequently among ESBL than non-ESBL isolates; however, the association was not significant in any of them. Within the ESBL group, class 1 genetic elements were associated with the bla_{CTX} genotype alone or in combination with bla_{TEM} in *E. coli* (36.58%), whereas this association was observed in 51.11% of *K. pneumoniae* isolates harboring bla_{SHV} and bla_{TEM} genotypes alone or in combination. In *E. coli*, no integrons were found in isolates containing bla_{SHV}. Similar results were observed for class 2 integrons. Class 3 integrons were not detected in any of the isolates studied, irrespective of being ESBL- or non-ESBL-producers. The simultaneous presence of class 1 and 2 integrons was detected more often in ESBL-producing than in non-ESBL-producing *E. coli* and *K. pneumoniae* isolates.

In order to observe associations between resistance patterns and integrons, we used a univariate analysis, which showed a highly significant association ($P < 0.001$) between MDR *E. coli* and *K. pneumoniae* and the presence of class 1 integrons. Remarkably, class 2 genetic elements were observed in two and seven MDR *E. coli* and *K. pneumoniae* isolates, respectively, that harbored quinolone resistance with the gyr and *parC* genes.

The associations between ciprofloxacin and nalidixic acid resistance, ESBL production, and the presence of

Table 2. ESBL Genotypes and Antibiotic Resistance Phenotypes Connotation

ESBL Genotype	Antibiotic Resistance Pattern	No. of Isolates (*E. coli* + *K. pneumoniae*)
bla_{TEM}	CAZ-CFM-CTR-CZ-GA-COT	10
	CAZ-CFM-CTR-CZ	9
	CAZ-CFM-CTR-CZ-GA	8
	CAZ-CFM-CTR-CZ-AK-GA	8
	CAZ-CFM-CTR-CZ-COT	7
	CAZ-CFM-CTR-CZ-AK-GA-IMI-COT	5
	CAZ-CFM-CTR-CZ-AK-GA -COT	3
	CAZ-CFM-CTR	3
bla_{SHV}	CAZ-CFM-CTR-CZ -GA- COT	13
	CAZ-CFM-CTR-CZ	11
	CAZ-CFM-CTR-CZ-AK-GA -COT	9
	CAZ-CFM-CTR-CZ -GA	5
	CAZ-CFM-CTR-CZ-AK-GA-IMI-COT	4
$bla_{CTX\text{-}M\text{-}3}$	CAZ-CFM-CTR-CZ- GA- COT	20
	CAZ-CFM-CTR-CZ-AK-GA- COT	9
	CFM-CTR-CZ-AK-GA-IMI-COT	7
$bla_{CTX\text{-}M\text{-}3}$ + TEM	CAZ-CFM-CTR-CZ- GA- COT	4
	CFM- CZ	3
	CAZ-CFM-CTR-CZ-AK-GA- COT	3
	CAZ-CFM-CTR-CZ- -COT	3
	CAZ-CFM-CTR-CZ-AK-GA-IMI-COT	2
	CAZ-CFM-CTR-CZ -GA	2
bla_{SHV} + TEM	CAZ-CFM-CTR	3
	CAZ-CFM-CTR-CZ- GA -COT	2
$bla_{CTX\text{-}M\text{-}3}$ + SHV	CAZ-CFM-CTR-CZ- GA	4
	CAZ-CFM-CTR-CZ-AK-GA- COT	3
	CAZ-CFM-CTR-CZ- GA -COT	3
	CFM-CTR-CZ-AK-GA -COT	2
	CAZ-CFM-CTR-CZ	2
$bla_{CTX\text{-}M\text{-}3}$ + SHV + TEM	CAZ-CFM-CTR-CZ	7
	CAZ-CFM-CTR-CZ-AK-GA -COT	4
	CAZ-CFM-CTR-CZ- GA- COT	4
	CAZ-CFM-CTR-CZ-AK-GA-IMI-COT	2

Table 3. Associations Between Antibiotics and ESBL Genotypes[a]

Genotype	Gentamicin	Amikacin	Imipenem	Co-Trimoxazole	Ceftazidime
bla_{TEM}	19 (46.3)	7 (46.6)	5 (41.6)	18 (47.3)	27 (49)
bla_{SHV}	10 (23)	9 (60)	6 (50)	19 (47.5)	24 (45)
$bla_{CTX\text{-}M\text{-}3}$	30 (73)	13 (86.6)	10 (83.3)	28 (73.6)	34 (61)
bla_{TEM}	13 (52)	6 (66.7)	2 (100)	17 (62.9)	24 (60)
bla_{SHV}	18 (72)	8 (88.8)	2 (100)	18 (66.7)	30 (75)
$bla_{CTX\text{-}M\text{-}3}$	17 (68)	8 (88.8)	2 (100)	18 (66.7)	25 (62)

[a] Values are expressed as No. (%).

class 1 or 2 integrons are shown in Table 4. Overall, just over half (50.7%) of ESBL producers, irrespective of genus, were simultaneously resistant to quinolones. The simultaneous presence of class 1 and 2 integrons in ciprofloxacin- and nalidixic-acid-resistant strains was the most frequent observation. The ciprofloxacin- and

nalidixic-acid-susceptible isolates did not carry class 1 or 2 integrons.

5. Discussion

Burn patients are at risk not only for wound infections, but also for other clinical complications due to a debilitated immunologic status. Clinical specimens may be obtained from various sites and sources of infection. Bloodstream infections and the subsequent development of sepsis are among the most common infection complications that occur following thermal injuries. These patients may also develop urinary tract infections associated with prolonged bladder catheterization (1, 2, 23, 24). Emerging antimicrobial resistance trends in bacterial pathogens represent a serious therapeutic challenge for the clinicians who care for these patients (25).

In the present study, *E. coli* was the predominant urinary tract pathogen and *K. pneumoniae* was an important wound pathogen. Mokaddas et al. (26) reported that burn patients are at high risk for nosocomial infections due to MDR bacteria, a large proportion of which are Gram-negative. The report from a 6-year review of bacteria identification and antibiotic susceptibility records at the US Army institute of surgical research burn center stressed the shifting epidemiology of bacterial isolates recovered during extended hospitalizations, and revealed that the bacteriology of burns begins with Gram-positive organisms or enteric bacteria (27).

The widespread use of β-lactam antibiotics to treat human infections may be associated with the selection of antibiotic resistance mechanisms in pathogenic and non-pathogenic isolates of *Enterobacteriaceae* (28). During the last few decades, resistance of Gram-negative bacilli to cephalosporin antibiotics has accelerated due to the appearance of ESBLs in *Klebsiella*, *E. coli*, and Proteus mirabilis. Thus, most clinicians have relied on imipenem, ciprofloxacin, or amikacin for the effective treatment of serious infections due to MDR *Klebsiella* (29).

The present study observed that 88.8% of *K. pneumoniae* and 57.7% of *E. coli* isolates were not susceptible to ceftazidime, the most common cephalosporin used in our hospital setting. Among the β-lactams, more than 80% of *E. coli* and *K. pneumoniae* isolates were resistant to cefazolin, cefotaxime, cefazolin, and ceftriaxone. Among the non-β-lactams, approximately 96% of *K. pneumoniae* were resistant to ciprofloxacin and 84% to ofloxacin, compared to 78% of *E. coli* isolates being resistant to ciprofloxacin and 61% to ofloxacin. Another study conducted to compare the susceptibility of ESBL-producing *Enterobacteriaceae* isolates with that of non-ESBL-producers found that approximately 40% were resistant to trimethoprim-sulfamethoxazole, 30% to ciprofloxacin, 30% to gentamicin, and 15% to piperacillin-tazobactam; it was suspected that these high levels of resistance to non-β-lactams were associated with the presence of ESBLs (30). The occurrence and distribution of ESBLs varies among different species and countries, demonstrating important geographical differences, and clinical specimens even vary between various hospital wards and between inpatients and outpatients. Zaniani and coworkers reported that the prevalences of ESBL-producing *E. coli* and *K. pneumoniae* isolates were 15.62% and 20%, respectively, in Iran (31). Research from India identified ESBLs in 70% - 90% of *Enterobacteriaceae* isolates in Pakistan, India, and the United Kingdom (32). Similar frequencies have been reported in many European countries (33-35), although published reports from some countries, including France and Canada, reveal a much lower prevalence (10% - 40%) (36, 37). A study from the United Arab Emirates (38) reported ESBL production in 39% of *E. coli* and 42% of *K. pneumonia* isolates. Published research from Turkey showed 12% of *E. coli* and 47% of *K. pneumoniae* isolates to be ESBL-positive (39). None of these studies were performed on burn patients.

In Iran, research has focused on ESBL production in *Acinetobacter baumannii* and *Pseudomonas aeruginosa*, and lacunae exist in information on the actual prevalence of ESBL-producing *E. coli* and *K. pneumoniae* isolates from burn patients. Our study is one of the first to investigate these two isolates from burn patients. We found *K. pneumoniae* and *E. coli* to be ESBL producers at rates of 71.4% and 57.7%, respectively, which is quite high. This was more pronounced by the presence of three ESBL genes, bla_{TEM}, bla_{SHV}, and bla_{CTX}, in these isolates. SHV and TEM ESBLs are derivatives of the narrow-spectrum SHV-1 and TEM-1/2 enzymes, while CTX-M enzymes are named for their strong hydrolytic activity for cefotaxime; they include the CTX-M-9, CTX-M-3, CTX-M-14, and CTX-M-15 families (40). In the present investigation, approximately 84% of *K. pneumoniae* and 92% of *E. coli* isolates were resistant to cefotaxime, and 58% and 67% were carriers of $bla_{\text{CTX-M-15}}$, respectively. The high prevalence of these β-lactamase enzymes suggests a serious problem, and drives a greater reliance on carbapenems.

The present study found class 1 genetic elements to be associated with 36.58% of *E. coli* isolates that produce ESBLS and with 51.11% of *K. pneumoniae* isolates harboring ESBL enzymes. Other interesting features were the significant associations between MDR patterns and the presence of class 1 integrons and class 2 genetic elements in few MDR *E. coli* and *K. pneumoniae* isolates harboring quinolone resistance with the *gyr* and *parC* genes. Broad antibiotic resistance extending to multiple antibiotic classes is now a frequent characteristic of ESBL-producing enterobacterial isolates.

Table 4. Association of Quinolone Resistance With ESBL Production, Carriage of Integron, and MDR

	Ciprofloxacin Resistance	Nalidixic Acid Resistance
ESBL producers	37 (50.7)	50 (68.4)
Non-ESBL producers	11 (45.8)	20 (83)
Presence ofintegrons		
Class 1	15 (45.4)	22 (66.6)
Class 2	8 (50)	13 (81.2)
Both class 1 and 2	10 (77)	11 (84.6)
Absence of integrons	15 (43)	24 (68)
MDR positive	38 (50)	57 (75)
MDR negative	10 (47.6)	13 (61.9)

Abbreviations: ESBL, extended-spectrum β-lactamase; MDR, multidrug resistance.

Interestingly, our study found the majority of *K. pneumoniae* and *E. coli* isolates to be significantly more resistant to ciprofloxacin and ofloxacin, leaving no options for treatment other than imipenem or amikacin. The MIC50 and MIC90 values for nalidixic acid for all *E. coli* and *K. pneumoniae* isolates were found at the resistance breakpoints (both = 163.55 mg/L), whereas for ciprofloxacin, MIC50 and MIC90 was 24.78 mg/L, indicating a major intervention in therapeutic management.

In the present study, the predominant ESBL genotype in *E. coli* was $bla_{\text{CTX-M-3}}$ (67.60%), while bla_{SHV} was the predominant (80.95%) and significant ($P < 0.001$) genotype in *K. pneumoniae*. Khosravi et al. (41) found SHV-1 to be the most prevalent ESBL gene, followed by TEM-1. It was also remarkable in our study that three β-lactamase genes were co-expressed in ESBL producers, and the most common combination comprised bla_{TEM} + $_{\text{SHV}}$+$_{\text{CTX}}$ and bla_{CTX} with bla_{TEM}. DNA sequence analyses of the bla_{TEM} sequences indicated that these two bacteria harbored the TEM-1b subgroup, whereas the subgroup found in the bla_{SHV} isolates was SHV-1. In the case of the $bla_{\text{CTX-M-3}}$ family, these isolates were carriers of the subgroup CTX-M-15. Moosavian and Deiham (42) studied the distribution of TEM, SHV, and CTX-M genes among ESBL-producing *Enterobacteriaceae* isolates in Iran (from patients outside of burn wards), but could not detect isolates carrying CTX-M-type ESBLs.

Hyle et al. (43) performed a multivariable analysis and found the infecting pathogen (*K. pneumoniae*) to be the only independent risk factor for MDR ESBL infections. We did not study the risk factors of ESBL production; however, the most frequent phenotypic pattern of MDR in our study was CAZ-CFM-CTR-CZ-GA-COT, which was found in 54.65% of isolates of ESBL-producing *E. coli* and *K. pneumoniae*, followed by CAZ-CFM-CTR-CZ-AK-GA-COT and CAZ-CFM-CTR-CZ, each observed in 33.72% of isolates. Our patients most likely acquired their infections through contact with colonized healthcare workers or contaminated fomites, or the isolates emerged as a result of the selective effect of antibiotic use. This is a prospective area that should be considered in the future.

5.1. Conclusions

The higher rate of ESBL production in *K. pneumoniae* and *E. coli* isolates shows that these are common in our hospital burn unit, with resistance to many classes of antibiotics, including fluoroquinolones, resulting in limited treatment options. MDR was common in *E coli* and *K. pneumoniae* isolates expressing ESBLs. In particular, the co-presence of three ESBL genotypes was associated with three- and four-class MDR. The transferable nature of these resistance genes is particularly worrisome and creates a demand for epidemiological studies and for improved infection-control procedures with a better understanding of the means by which spread occurs.

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Footnotes

Author's Contribution: All authors contributed equally to this work. Study concept and design: Alka Hasani

and Ali Purmohammad; analysis and interpretation of data: Alka Hasani and Ali Purmohammad; drafting of the manuscript: Alka Hasani, Ali Purmohammad, and Mohammad Ahangarzadeh Rezaee; critical revision of the manuscript for important intellectual content: Alka Hasani, Ali Purmohammad, Mohammad Ahangarzadeh Rezaee, and Akbar Hasani; statistical analysis: Alka Hasani and Akbar Hasani.

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References

1. Pruitt BJ, McManus AT. The changing epidemiology of infection in burn patients. *World J Surg.* 1992;**16**(1):57–67. [PubMed: 1290268].
2. Church D, Elsayed S, Reid O, Winston B, Lindsay R. Burn wound infections. *Clin Microbiol Rev.* 2006;**19**(2):403–34. doi: 10.1128/CMR.19.2.403-434.2006. [PubMed: 16614255].
3. Kurioka A, Ussher JE, Cosgrove C, Clough C, Fergusson JR, Smith K, et al. MAIT cells are licensed through granzyme exchange to kill bacterially sensitized targets. *Mucosal Immunol.* 2015;**8**(2):429–40. doi: 10.1038/mi.2014.81. [PubMed: 25269706].
4. Bennett JW, Robertson JL, Hospenthal DR, Wolf SE, Chung KK, Mende K, et al. Impact of extended spectrum beta-lactamase producing Klebsiella pneumoniae infections in severely burned patients. *J Am Coll Surg.* 2010;**211**(3):391–9. doi: 10.1016/j.jamcollsurg.2010.03.030. [PubMed: 20800197].
5. Pakzad I, Karin MZ, Taherikalani M, Boustanshenas M, Lari A. Contribution of AcrAB efflux pump to ciprofloxacin resistance in Klebsiella pneumoniae isolated from burn patients. *GMS Hygiene Infect Control.* 2013;**8**(2).
6. Lagace-Wiens PR, Nichol KA, Nicolle LE, Decorby MR, McCracken M, Alfa MJ, et al. ESBL genotypes in fluoroquinolone-resistant and fluoroquinolone-susceptible ESBL-producing Escherichia coli urinary isolates in Manitoba. *Can J Infect Dis Med Microbiol.* 2007;**18**(2):133–7. [PubMed: 18923714].
7. Lautenbach E, Patel JB, Bilker WB, Edelstein PH, Fishman NO. Extended-spectrum beta-lactamase-producing Escherichia coli and Klebsiella pneumoniae: risk factors for infection and impact of resistance on outcomes. *Clin Infect Dis.* 2001;**32**(8):1162–71. doi: 10.1086/319757. [PubMed: 11283805].
8. Lautenbach E, Strom BL, Bilker WB, Patel JB, Edelstein PH, Fishman NO. Epidemiological investigation of fluoroquinolone resistance in infections due to extended-spectrum beta-lactamase-producing Escherichia coli and Klebsiella pneumoniae. *Clin Infect Dis.* 2001;**33**(8):1288–94. doi: 10.1086/322667. [PubMed: 11565067].
9. Garza-Gonzalez E, Mendoza Ibarra SI, Llaca-Diaz JM, Gonzalez GM. Molecular characterization and antimicrobial susceptibility of extended-spectrum {beta}-lactamase-producing Enterobacteriaceae isolates at a tertiary-care centre in Monterrey, Mexico. *J Med Microbiol.* 2011;**60**(Pt 1):84–90. doi: 10.1099/jmm.0.022970-0. [PubMed: 20930052].
10. Machado E, Canton R, Baquero F, Galan JC, Rollan A, Peixe L, et al. Integron content of extended-spectrum-beta-lactamase-producing Escherichia coli strains over 12 years in a single hospital in Madrid, Spain. *Antimicrob Agents Chemother.* 2005;**49**(5):1823–9. doi: 10.1128/AAC.49.5.1823-1829.2005. [PubMed: 15855502].
11. Rao AN, Barlow M, Clark LA, Boring J3, Tenover FC, McGowan JJ. Class 1 integrons in resistant Escherichia coli and Klebsiella spp., US hospitals. *Emerg Infect Dis.* 2006;**12**(6):1011–4. [PubMed: 16707065].
12. Fallah F, Karimi A, Goudarzi M, Shiva F, Navidinia M, Jahromi MH, et al. Determination of integron frequency by a polymerase chain reaction-restriction fragment length polymorphism method in multidrug-resistant Escherichia coli, which causes urinary tract infections. *Microb Drug Resist.* 2012;**18**(6):546–9. doi: 10.1089/mdr.2012.0073. [PubMed: 22816551].
13. Forbes BA, Sahm DF, Weissfeld AS. In: Bailey & Scott's Diagnostic Microbiology. Patricia M, editor. Elsevier Mosby; 2014. pp. 307–15.Enterobacteriaceae.
14. McCartney JE, Collee JG, Mackie TJ. Practical Medical Microbiology. Charchil Livingstone; 1989.
15. Clinical and Laboratory Standards Institute . Performance standards for antimicrobial susceptibility testing; twenty-first informational supplements M100-S21. Wayne: Clinical and Laboratory Standards Institute; 2011.
16. Sambrook J, Fritsch EF, Maniatis T. Molecular cloning: A laboratory manual+ Cold Spring Harbor. New York: Cold Spring Harbor Laboratory Press; 1989.
17. Sompolinsky D, Nitzan Y, Tetry S, Wolk M, Vulikh I, Kerrn MB, et al. Integron-mediated ESBL resistance in rare serotypes of Escherichia coli causing infections in an elderly population of Israel. *J Antimicrob Chemother.* 2005;**55**(1):119–22. doi: 10.1093/jac/dkh517. [PubMed: 15574469].
18. Hanson ND, Thomson KS, Moland ES, Sanders CC, Berthold G, Penn RG. Molecular characterization of a multiply resistant Klebsiella pneumoniae encoding ESBLs and a plasmid-mediated AmpC. *J Antimicrob Chemother.* 1999;**44**(3):377–80. [PubMed: 10511405].
19. Pagani L, Dell'Amico E, Migliavacca R, D'Andrea MM, Giacobone E, Amicosante G, et al. Multiple CTX-M-type extended-spectrum beta-lactamases in nosocomial isolates of Enterobacteriaceae from a hospital in northern Italy. *J Clin Microbiol.* 2003;**41**(9):4264–9. [PubMed: 12958255].
20. Bansal S, Tandon V. Contribution of mutations in DNA gyrase and topoisomerase IV genes to ciprofloxacin resistance in Escherichia coli clinical isolates. *Int J Antimicrob Agents.* 2011;**37**(3):253–5. doi: 10.1016/j.ijantimicag.2010.11.022. [PubMed: 21236644].
21. Vasilaki O, Ntokou E, Ikonomidis A, Sofianou D, Frantzidou F, Alexiou-Daniel S, et al. Emergence of the plasmid-mediated quinolone resistance gene qnrS1 in Escherichia coli isolates in Greece. *Antimicrob Agents Chemother.* 2008;**52**(8):2996–7. doi: 10.1128/AAC.00325-08. [PubMed: 18490503].
22. Shibata N, Doi Y, Yamane K, Yagi T, Kurokawa H, Shibayama K, et al. PCR typing of genetic determinants for metallo-beta-lactamases and integrases carried by gram-negative bacteria isolated in Japan, with focus on the class 3 integron. *J Clin Microbiol.* 2003;**41**(12):5407–13. [PubMed: 14662918].
23. Navidinia M, Peerayeh SN, Fallah F, Bakhshi B, Sajadinia RS. Phylogenetic grouping and pathotypic comparison of urine and fecal Escherichia coli isolates from children with urinary tract infection. *Braz J Microbiol.* 2014;**45**(2):509–14. [PubMed: 25242935].
24. Navidinia M, Peerayeh SN, Fallah F, Bakhshi B, Adabian S, Alimehr S, et al. Distribution of the Pathogenicity Islands Markers (PAIs) in Uropathogenic E. coli Isolated from Children in Mofid Children Hospital. *Arch Pediatr Infect Dis.* 2013;**1**(2):75–9.
25. Murphy KD, Lee JO, Herndon DN. Current pharmacotherapy for the treatment of severe burns. *Expert Opin Pharmacother.* 2003;**4**(3):369–84. doi: 10.1517/14656566.4.3.369. [PubMed: 12614189].
26. Mokaddas E, Rotimi VO, Sanyal SC. In vitro activity of piperacillin/tazobactam versus other broad-spectrum antibiotics against nosocomial gram-negative pathogens isolated from burn patients. *J Chemother.* 1998;**10**(3):208–14. doi: 10.1179/joc.1998.10.3.208. [PubMed: 9669645].
27. Keen E3, Robinson BJ, Hospenthal DR, Aldous WK, Wolf SE, Chung KK, et al. Incidence and bacteriology of burn infections at a military burn center. *Burns.* 2010;**36**(4):461–8. doi: 10.1016/j.burns.2009.10.012. [PubMed: 20045259].

28. Brinas L, Zarazaga M, Saenz Y, Ruiz-Larrea F, Torres C. Beta-lactamases in ampicillin-resistant Escherichia coli isolates from foods, humans, and healthy animals. *Antimicrob Agents Chemother.* 2002;**46**(10):3156–63. [PubMed: 12234838].
29. Rahal JJ, Urban C, Horn D, Freeman K, Segal-Maurer S, Maurer J, et al. Class restriction of cephalosporin use to control total cephalosporin resistance in nosocomial Klebsiella. *JAMA.* 1998;**280**(14):1233–7. [PubMed: 9786372].
30. Schwaber MJ, Navon-Venezia S, Schwartz D, Carmeli Y. High levels of antimicrobial coresistance among extended-spectrum-beta-lactamase-producing Enterobacteriaceae. *Antimicrob Agents Chemother.* 2005;**49**(5):2137–9. doi: 10.1128/AAC.49.5.2137-2139.2005. [PubMed: 15855548].
31. Riyahi Zaniani F, Meshkat Z, Naderi Nasab M, Khaje-Karamadini M, Ghazvini K, Rezaee A, et al. The prevalence of TEM and SHV genes among extended-spectrum beta-lactamases producing Escherichia coli and Klebsiella pneumoniae. *Iranian J Basic Med Sci.* 2012;**15**(1):654–60.
32. Kumarasamy KK, Toleman MA, Walsh TR, Bagaria J, Butt F, Balakrishnan R, et al. Emergence of a new antibiotic resistance mechanism in India, Pakistan, and the UK: a molecular, biological, and epidemiological study. *Lancet Infect Dis.* 2010;**10**(9):597–602. doi: 10.1016/S1473-3099(10)70143-2. [PubMed: 20705517].
33. Coque TM, Baquero F, Canton R. Increasing prevalence of ESBL-producing Enterobacteriaceae in Europe. *Eur Commun Dis Bull.* 2008;**13**(47):5437–53.
34. Pitout JD, Nordmann P, Laupland KB, Poirel L. Emergence of Enterobacteriaceae producing extended-spectrum beta-lactamases (ESBLs) in the community. *J Antimicrob Chemother.* 2005;**56**(1):52–9. doi: 10.1093/jac/dki166. [PubMed: 15917288].
35. Paterson DL, Bonomo RA. Extended-spectrum beta-lactamases: a clinical update. *Clin Microbiol Rev.* 2005;**18**(4):657–86. doi: 10.1128/CMR.18.4.657-686.2005. [PubMed: 16223952].
36. Rupp ME, Fey PD. Extended spectrum β-lactamase (ESBL)-producing Enterobacteriaceae. *Drugs.* 2003;**63**(4):353–65.
37. Spanu T, Luzzaro F, Perilli M, Amicosante G, Toniolo A, Fadda G, et al. Occurrence of extended-spectrum beta-lactamases in members of the family Enterobacteriaceae in Italy: implications for resistance to beta-lactams and other antimicrobial drugs. *Antimicrob Agents Chemother.* 2002;**46**(1):196–202. [PubMed: 11751134].
38. Al-Zarouni M, Senok A, Rashid F, Al-Jesmi SM, Panigrahi D. Prevalence and antimicrobial susceptibility pattern of extended-spectrum beta-lactamase-producing Enterobacteriaceae in the United Arab Emirates. *Med Princ Pract.* 2008;**17**(1):32–6. doi: 10.1159/000109587. [PubMed: 18059098].
39. Ozgunes I, Erben N, Kiremitci A, Kartal ED, Durmaz G, Colak H, et al. The prevalence of extended-spectrum beta lactamase-producing Escherichia coli and Klebsiella pneumoniae in clinical isolates and risk factors. *Saudi Med J.* 2006;**27**(5):608–12. [PubMed: 16680246].
40. Bonnet R. Growing group of extended-spectrum beta-lactamases: the CTX-M enzymes. *Antimicrob Agents Chemother.* 2004;**48**(1):1–14. [PubMed: 14693512].
41. Khosravi AD, Hoveizavi H, Mehdinejad M. Prevalence of Klebsiella pneumoniae encoding genes for CTX-M-1, TEM-1 and SHV-1 extended-spectrum beta lactamases (ESBL) enzymes in clinical specimens. *Jundishapur J Microbiol.* 2013;**6**(10).
42. Moosavian M, Deiham B. Distribution of TEM, SHV and CTX-M Genes among ESBL-producing Enterobacteriaceae isolates in Iran. *Afr J Microbiol Res.* 2012;**6**(26):5433–9.
43. Hyle EP, Lipworth AD, Zaoutis TE, Nachamkin I, Fishman NO, Bilker WB, et al. Risk factors for increasing multidrug resistance among extended-spectrum beta-lactamase-producing Escherichia coli and Klebsiella species. *Clin Infect Dis.* 2005;**40**(9):1317–24. doi: 10.1086/429239. [PubMed: 15825035].