Research Article

Detection of extended-spectrum betalactamase and carbapenemase-producing Enterobacteriaceae in Tunisia

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Abstract

The emergence of dramatic urinary tract infections (UTIs) caused by the members of the Enterobacteriales is an important public health problem in the community as well as in Tunisian hospitals. This study aims to investigate the prevalence of extended-spectrum β-lactamase (ESBL) and carbapenemase-producing uropathogenic isolates of Escherichia coli (E. coli) and Klebsiella pneumoniae (K. pneumoniae).

Based on decreased susceptibility to β-lactams antibiotics and analyzed for the presence of ESBL and carbapenemase genes by Real Time-polymerase chain reaction (RT-PCR), 56 uropathogenic isolates of E. coli (n = 36) and K. pneumoniae (n = 20) were confirmed positive for ESBLs. The CTX-M-type β-lactamases were mostly detected in E. coli isolates (21 strains, 58.33% [95% CI 38.09% - 72.06%]) followed by blαTEM-like (14 strains, 50% [95% CI 32.92% - 67.07%]), blαSHV-like and blαOXA-48-like simultaneously (15 strains, 41.67% [95% CI 25.51% - 59.24%]). Furthermore, the RT-PCR system on the K. pneumoniae strains demonstrated that blαSHV-12-like was the most predominant (16 strains, 80% [95% CI 56.33% - 94.26%]) followed by blαTEM-like (14 strains, 70% [95% CI 45.72% - 88.10%]), blαCTX-M belonging to groups 9 and 1 (11 strains, 55% [95% CI 31.52% - 76.94%]) and finally blαOXA-48-like (10 strains, 50% [95% CI 27.19% - 72.80%]). In addition, E. coli and K. pneumoniae strains harbored a carbapenemase gene blαDHD-2αes with 22.2% [95% CI 10.11% - 39.15%]; 20% [95% CI 12.83% - 43.66%], respectively.

Our results confirm the need to monitor the resistance to extended-spectrum β-lactams and to carbapenems among enterobacteria in Tunisia.

Introduction

Urinary tract infections (UTIs) are a real public health problem both in terms of frequency and difficulty of treatment of multidrug-resistant bacteria, as well as of β-lactamase and carbapenemase-producing bacteria [1]. Extended-spectrum β-lactamase (ESBL) and Carbapenemase-producing Enterobacteriaceae (CPE) strains have been increasingly reported in Europe, South America, Asia, Oceania, and Africa in recent years [2]. However, E. coli and K. pneumoniae are also two of the most common pathogenic bacteria causing urinary tract and bloodstream infections by means of a remarkable range of virulence factors that can affect a wide variety of host cell processes [3]. The indiscriminate use of antibiotics has contributed to their significant presence in the environment, promoting resistant microorganisms by selection, mutation, and recombination through horizontal transfers [4]. The massive use of C3G antibiotics in the treatment of bacterial infections has contributed to the worldwide spread of Enterobacteriales, which produce ESBL [5]. In Tunisia, resistance to third-generation cephalosporins (C3G) has increased lately. In fact, of the total isolates of uropathogenic Enterobacteriales, it has been reported that 6.2% were...
resistant to C3G in 2012 and 19.7% in 2017 (https://www.infectiologie.org.tn/resistance.php). Until the late 1990s, the detected ESBLs were derived from the old narrow-spectrum penicillinas TEM-1, TEM-2, and SHV-1. Subsequently, other types of ESBLs have emerged as CTX-M, PER, VEB, and GES [6,7]. The CTX-M enzymes confer a high level of resistance to cefotaxime, although some variants, such as CTX-M-15, have also strong activity against ceftazidime [8]. Nowadays, Enterobacteriales may also express ESBLs that are not closely connected to TEM- or SHV-related species, including CTX-M and OXA-type ESBLs [9]. Currently, carbapenems are the most powerful agents prescribed for the treatment of serious infections caused by Enterobacteriales species given their broad spectrum of antimicrobial activity and excellent resistance to hydrolysis via most of the extended-spectrum $\beta$-lactamases (ESBLs) and cephalosporinases [10].

The main mechanism behind carbapenem resistance is the acquisition of OXA-48-like carbapenemases is an oxacillinase that is from the clinical isolate of K. pneumoniae in Turkey in 2001 [9,11]. These ESBLs are typically plasmid-mediated rather than chromosomally mediated $\beta$-lactamases [12]. Indeed, they have broader-range activity, covering carbapenems as well as extended-spectrum cephalosporins [13,14]. The resistance to broad-spectrum cephalosporins has increased among Enterobacteriales strains from both human and animal sources [15]. Although Hall, et al. indicate that food might be a source of human-acquired antimicrobial-resistant E. coli due to the fact that similar ESBLs and plasmids encoding them have been detected in food-producing animals, food of animal origin, and humans [16]. In response to the emergence of ESBLs and carbapenemases in Tunisia [17], in addition to the misuse of antimicrobial agents, the launch of empirical treatment of UTIs cases is often based on the antimicrobial resistance pattern of the urinary pathogens from existing surveillance report [18]. Therefore, this study aims at developing an RT-PCR system for the detection of clinic blaTEM, blaSHV, blaCTX-M group-9, blaCTX-M group-2, and blaOXA-48 group genes in order to apply it in clinical E. coli and K. pneumoniae isolates collected from Tunisian hospitals. Herein, we describe the spread of E. coli and K. pneumoniae–harbored blaOXA-48 and ESBL-encoding genes in Tunisian hospitals.

### Materials and methods

#### Collection and identification of Enterobacteriales strains

Urine samples were collected in sterile containers using aseptic techniques from patients aged between 25 and 55 years and suspected to have UTIs. Of the total 1600 urine samples collected between January 2018 and March 2018, only 200 positive urine cultures were selected through random sampling. The selected urine samples were taken from patients of healthcare facilities and from community patients located in the city of Sfax and Tunis.

ESBL-producing Enterobacteriaceae (ESBL-E) identification was accomplished via conventional methods, including biochemical tests (BioMerieux API 10S) [19] and confirmed using Matrix-Assisted Laser Desorption Ionization-Time-Of-Flight/Mass Spectrometry (MALDI-TOF/MS, Bruker Daltonics).

#### Antibiotic susceptibility testing

All 176 Enterobacteriaceae isolates were tested for antimicrobial susceptibility using the Mueller-Hinton (MH) agar (Bio-Rad) disk-diffusion technique. The susceptibility was tested to the following antibiotics: Amoxicillin-clavulanic acid (AMC), Ticaricillin (TIC), Cefotaxime (CTX), Ceftazidime (CAZ), Cefalotin (CF), Cefixime (CFM), Imipenem (IMP), Amikacin (AN), Gentamicin (GM), Netilmicin (NET), Tobramycin (NN), Nalidixic acid (NA), Ofloxacin (OFX), Ciprofloxacin (CIP), Fosfomycin (FFL), Nitrofurantoin (FM), Trimethoprim-sulfamethoxazole (SXT), Chloramphenicol (C). Inhibition zone diameters were interpreted according to Clinical and Laboratory Standards Institute recommendations and E. coli ATCC 25922 was used as a quality control strain [20].

#### Double disc synergy test

The isolates collected were distributed on an MH agar plate. The two antibiotic disks of CTX (30 μg) and CAZ (30 μg) were placed at a distance of 25 mm (edge to edge) from the AMC (20/10 μg) disc that was placed in the middle of the plate [21].

After a 24-h incubation, a test was considered positive if an enhanced zone of inhibition between either of the cephalosporin antibiotics and the AMC disc occurred. This indicated synergistic activity with Clavulanic acid and the presence of an ESBL [22].

#### Detection of extended-spectrum $\beta$-lactamase genes using Real-time PCR

The presence of different Ambler class A $\beta$-lactamase encoding genes has been tested through RT-PCR using specific primers for bla_{CTX-M}, bla_{SHV} and bla_{TEM} [19]. Other $\beta$-lactamase encoding genes, belonging to class D (bla_{OXA-48-like}) and class C (bla_{CMY-2-like}), have also been tested as previously described [23,24].

RT-PCR was performed for beta-lactamase genes of the family TEM-like, SHV-like, and CTX-M of the two most prevalent groups, ie, CTX-M of group 1 and group 9 (named CTX-M-G1 and CTX-M-G9, respectively), CMY-2-like, and OXA-48-like via specific primers, designed using Primer-BLAST (Table 1) Figure 1.

Monoplex PCR assays were optimized using Applied Biosystems including the Taq SYBR Green MasterMix (Invitrogen Life Technologies) and Microamp 96-Well Reaction Plate 0.1mL (Thermo-Fisher Scientific). Briefly, reactions were performed in a final volume of 20μL.
Detection of extended-spectrum betalactamase and carbapenemase-producing Enterobacteriaceae in Tunisia

Figure 1: Results of real time PCR for blaSHV-12-like/blaTEM-like/blaCTX-M-G1-like/blaCTX-M-G9/blaCMY-2-like/blaOXA-48-like (a1), (a2), (a3), (a4), (a5), (a6): the profile of DNA amplification for blaSHV-12-like, blaTEM-like, blaCTX-M-G1, blaCTX-M-G9, blaCMY-2-like, blaOXA-48-like respectively. (b1), (b2), (b3), (b4), (b5), (b6): the profile of melt curve for blaSHV-12-like (81), blaTEM-like (85.5), blaCTX-M-G1 (83), blaCTX-M-G9 (85), blaCMY-2-like (80.5), blaOXA-48-like (84.5) respectively.
containing 100nM DNA solution and 10 μmol of each gene-specific primer. PCR amplification conditions were as follows: initial denaturation step at 95 °C for 3 min; 45 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, extension at 72 °C for 2 min, followed by a final extension step at 72 °C for 10 min. Amplification results were analyzed with the threshold and baseline set between 10 and 25 cycles. Melting-curve analysis was performed as follows: 60 °C for 3s (ramp rate = 5 °C/s) until reaching a final temperature of 95 °C, with fluorescence fluctuation, analyzed during the latter. The number of DNA molecules present in the sample was determined based on the amount of fluorescence detected by the RT-PCR instrument [23]. This quantified fluorescence resulted in a Cycle Threshold value (Ct) that corresponds to the number of amplification cycles required to obtain a given amount of DNA [25].

Statistical analysis

Statistical tests including Spearman’s rank correlation analysis, χ2 test, and multiple logistic regression analysis were used to evaluate the associations between the determinant bla group genes and the various levels of antibiotic resistance phenotypes. The level of significance was set at p < 0.05 in this study. All statistical analyses were carried out using IBM SPSS Statistics for Windows, Version 16.0 (SPSS Inc., Chicago, IL, USA).

Results

Prevalence of ESBL-producing Enterobacteriaceae in Tunisian healthcare facilities

Of the 176 isolates that were classified as Enterobacteriales, 100 isolates (50%) were identified to be E. coli, which was predominant, followed by 50 isolates of K. pneumoniae (25%), 20 isolates of Enterobacter cloacae complex (10%) and 6 isolates of Citrobacter koseri (3%). Fifty-six isolates (28%), considered ESBL-producing, were initially classified as Enterobacter spp. through biochemical tests (API 10S gallery). Following matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS), those isolates were re-identified and confirmed as 36 strains of E. coli and 20 of K. pneumoniae.

Antibiotic resistance patterns

The antimicrobial susceptibility patterns were determined for all 176 Enterobacteriales urine isolates. After identification, the total of 56 ESBL-producing uropathogenic isolates included 36 strains of E. coli and 20 of K. pneumoniae.

The ESBL-producing E. coli and K. pneumoniae uropathogenic isolates showed higher levels of resistance to all antibiotics compared to the non-ESBL-producing isolates except for imipenem, since the majority of the isolates tested in this study were imipenem-sensitive. Antibiotic susceptibility testing for the 56 isolates is summarized in Table 2.

A high prevalence of resistance was observed against third-generation antibiotics (3GCs). All the isolates were resistant to AMX, TIC, CAZ, and CTX. Resistance to 3GCs was associated predominantly with the presence of blaCTX-M genes.

The 36 isolates of E. coli showed a high level of resistance to CIP (n = 34, 94.44% [CI 81.33% - 99.31%]), OFX (n = 33, 91.66% [CI 77.53% - 98.24%]), as well as a moderate level of resistance to imipenem (n = 7, 19.45% [CI 8.1% - 36%]) (Table 2). The 20 isolates of K. pneumoniae also showed a high level of resistance to members of the quinolone family, including CIP (n = 16, 80% [CI 56.33% - 94.26%]), OFX (n = 15, 75% [CI 50.89% - 91.34%]), in addition to imipenem which was reported on a level of four K. pneumoniae strains (20% [CI 5.73% - 43.66%]) (Table 2). All isolates were susceptible to colistin. The double-disc synergy test showed that 29% of the isolates were ESBL-positive.

All ESBL-producing E. coli strains showed a decreased susceptibility to β-lactams, except for cephapemycins (cefoxitin) and carbapenems. This latter remained susceptible to fosfomycin, and both were resistant to nitrofurantoin. All E. coli isolates were resistant to co-trimoxazole (sulfamethoxazole-trimethoprim). They exhibited different aminoglycoside phenotypes (resistance to gentamycin, tobramycin, and amikacin, 26%, 47%, and 20%, respectively) (Table 2). Among the K. pneumoniae strains, various resistance profiles were demonstrated to tested antibiotics (Table 2).
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Table 2: Antibiotic susceptibility profile of ESBL-producing E. coli and K. pneumoniae.

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>ESBL-producing E. coli</th>
<th>ESBL-producing K. pneumoniae</th>
</tr>
</thead>
<tbody>
<tr>
<td>Penicillin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AMC</td>
<td>100% (36) [95% CI 100% (20) 0</td>
<td></td>
</tr>
<tr>
<td>TIC</td>
<td>100% (36) [95% CI 100% (20) 0</td>
<td></td>
</tr>
<tr>
<td>Cephalosporin (1GCs)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CF</td>
<td>80.55% (29) [95% CI 72.22% (20) 0</td>
<td></td>
</tr>
<tr>
<td>Cephalosporin (3GCs)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CFM</td>
<td>72.22% (20) [95% CI 69.45% (25) 0</td>
<td></td>
</tr>
<tr>
<td>CTX</td>
<td>100% (36) [95% CI 100% (20) 0</td>
<td></td>
</tr>
<tr>
<td>CAZ</td>
<td>100% (36) [95% CI 100% (20) 0</td>
<td></td>
</tr>
<tr>
<td>Carbapenem</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IMP</td>
<td>19.45% (7) [95% CI 10-45% (14) 0</td>
<td></td>
</tr>
<tr>
<td>Fluoroquinolone</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OFX</td>
<td>91.66% (33) [95% CI 89.45% (26) 0</td>
<td></td>
</tr>
<tr>
<td>CIP</td>
<td>94.44% (34) [95% CI 87.22% (22) 0</td>
<td></td>
</tr>
<tr>
<td>AN</td>
<td>79.46% (17) [95% CI 70.42% (14) 0</td>
<td></td>
</tr>
<tr>
<td>GM</td>
<td>47.22% (17) [95% CI 35.76% (10) 0</td>
<td></td>
</tr>
<tr>
<td>NET</td>
<td>30.55% (11) [95% CI 25.72% (9) 0</td>
<td></td>
</tr>
<tr>
<td>NN</td>
<td>47.22% (17) [95% CI 35.76% (10) 0</td>
<td></td>
</tr>
<tr>
<td>FFL</td>
<td>5.56% (2) [95% CI 4.94% (2) 0</td>
<td></td>
</tr>
<tr>
<td>SXT</td>
<td>36.11% (13) [95% CI 26.83% (9) 0</td>
<td></td>
</tr>
<tr>
<td>FM</td>
<td>13.88% (5) [95% CI 10.46% (4) 0</td>
<td></td>
</tr>
</tbody>
</table>

β-Lactams

Antibiotics | Sensitive; [95% CI] | Sensitive; [95% CI] |
-------------|---------------------|---------------------|
Penicillin   | 100% (36) [95% CI 100% (20) 0 |                             |
Cephalosporin (1GCs) |                |              |
CFM          | 72.22% (20) [95% CI 69.45% (25) 0 |                             |
Cephalosporin (3GCs) |                |              |
CFM          | 72.22% (20) [95% CI 69.45% (25) 0 |                             |
CTX          | 100% (36) [95% CI 100% (20) 0 |                             |
CAZ          | 100% (36) [95% CI 100% (20) 0 |                             |
Carbapenem   | 19.45% (7) [95% CI 10-45% (14) 0 |                             |
Fluoroquinolone |                |              |
OFX          | 91.66% (33) [95% CI 89.45% (26) 0 |                             |
CIP          | 94.44% (34) [95% CI 87.22% (22) 0 |                             |
AN           | 79.46% (17) [95% CI 70.42% (14) 0 |                             |
GM           | 47.22% (17) [95% CI 35.76% (10) 0 |                             |
NET          | 30.55% (11) [95% CI 25.72% (9) 0 |                             |
NN           | 47.22% (17) [95% CI 35.76% (10) 0 |                             |
FFL          | 5.56% (2) [95% CI 4.94% (2) 0 |                             |
SXT          | 36.11% (13) [95% CI 26.83% (9) 0 |                             |
FM           | 13.88% (5) [95% CI 10.46% (4) 0 |                             |

Aminosides

Antibiotics | Sensitive; [95% CI] | Sensitive; [95% CI] |
-------------|---------------------|---------------------|
Penicillin   | 100% (36) [95% CI 100% (20) 0 |                             |
Cephalosporin (1GCs) |                |              |
CFM          | 72.22% (20) [95% CI 69.45% (25) 0 |                             |
Cephalosporin (3GCs) |                |              |
CFM          | 72.22% (20) [95% CI 69.45% (25) 0 |                             |
CTX          | 100% (36) [95% CI 100% (20) 0 |                             |
CAZ          | 100% (36) [95% CI 100% (20) 0 |                             |
Carbapenem   | 19.45% (7) [95% CI 10-45% (14) 0 |                             |
Fluoroquinolone |                |              |
OFX          | 91.66% (33) [95% CI 89.45% (26) 0 |                             |
CIP          | 94.44% (34) [95% CI 87.22% (22) 0 |                             |
AN           | 79.46% (17) [95% CI 70.42% (14) 0 |                             |
GM           | 47.22% (17) [95% CI 35.76% (10) 0 |                             |
NET          | 30.55% (11) [95% CI 25.72% (9) 0 |                             |
NN           | 47.22% (17) [95% CI 35.76% (10) 0 |                             |
FFL          | 5.56% (2) [95% CI 4.94% (2) 0 |                             |
SXT          | 36.11% (13) [95% CI 25.51% (9) 0 |                             |
FM           | 13.88% (5) [95% CI 10.46% (4) 0 |                             |

Prevalence of ESBL genes

The newly developed RT-PCR system was efficient in detecting the bl regarding group genes. All positive phenotypic ESBL and carbapenemase isolates were analyzed for the presence of genes encoding bl TEM-like, bl SHV- like, bl CTX-M-G1, bl CTX-M-G9, bl CMY-2-like and bl OXA-48-like. Further details on the results are shown in Tables 3 and 4.

The ESBL production was detected in all ceftazidime and cefotaxime-resistant E. coli and K. pneumonia strains recovered in this study. In terms of E. coli, bl TEM-like and bl CTX-M-G9 were the most prevalent (21 strains, 58.33% [95% CI 38.09% - 72.06%]) followed by bl SHV-like (18 strains, 50% [95% CI 32.92% - 67.07%]), bl TEM-like and bl CMY-2-like Simultaneously (15 strains, 41.67% [95% CI 25.51% - 59.24%]). Furthermore, the RT-PCR system on the K. pneumoniae strains demonstrated that bl SHV-like was the most predominant (16 strains, 80% [95% CI 56.33% - 94.26%]) followed by bl TEM-like (14 strains, 70% [95% CI 45.72% - 88.10%]), bl CTX-M-G9 and bl CTX-M-G1 (11 strains, 55% [95% CI 31.52% - 76.94%]), and finally bl CMY-2-like (10 strains, 50% [95% CI 27.19% - 72.80%]) (Tables 3 and 4).

On the other hand, 22.2% (8 strains) and 20% (4 strains) of ESBL-producing E. coli and K. pneumoniae strains, respectively, possessed at least one bl group gene with 6 different genotypes (Tables 3 and 4).

Besides all the third-generation cephalosporin-resistant strains, 14 of them (6 E. coli, 16.64% and 8 K. pneumoniae, 40%, respectively) harbored the four bls groups: i.e. bl TEM-like, bl SHV-like, and both bl CTX-M-G1 and bl CTX-M-G9.

Interestingly, the presence of the bl OXA-48-like gene was reported in eight isolates of E. coli [22.2% [95% CI 10.11% - 39.15%] and four isolates of K. pneumoniae [20% [95% CI 12.83% - 43.66%]] in this study. Indeed, seven E. coli and four K. pneumoniae isolates, resistant to imipenem, were found to produce OXA-48-like (Table 4). These data confirmed that the OXA-48-like gene was widespread in Tunisian healthcare facilities.

In conclusion, a high CTX-M prevalence was found in ESBL-producing strains of both E. coli and K. pneumonia. Nevertheless, all the OXA-48-like-producing isolates coproduced other β-lactamases such as TEM-like, SHV-like, CTX-M, and CMY-2-like (Tables 3 and 4).

Resistance to three or more β-lactam agents was associated with specific gene types and numbers

Resistance to three or more agents was associated with the presence of a specific gene type. Specifically, analysis of our data revealed that carriage of bl CMY-2-like and bl OXA-48-like genes correlated positively with resistance to three or more antibiotics (Po = 0.049 and 0.005, respectively; Table 5).
Table 3: Prevalence of blaTEM-like, blaSHV-like, blaCTX-M1, blaCTX-M9, blaCMY-2-like and blaOXA-48-like genes in ESBL producers for E.coli and K. pneumoniae.

<table>
<thead>
<tr>
<th>Identified genes</th>
<th>E.coli [95% CI]</th>
<th>K.pneumoniae [95% CI]</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHV-like</td>
<td>50%(32.92%-67.07%)</td>
<td>80%(56.33%-94.26%)</td>
</tr>
<tr>
<td>TEM-like</td>
<td>41.67%(25.51%-59.24%)</td>
<td>70%(45.72%-88.10%)</td>
</tr>
<tr>
<td>CTX-M-G9</td>
<td>58.33%(38.09%-72.06%)</td>
<td>55%(31.52%-76.94%)</td>
</tr>
<tr>
<td>CTX-M-G1</td>
<td>58.33%(38.09%-72.06%)</td>
<td>55%(31.52%-76.94%)</td>
</tr>
<tr>
<td>CMY-2-like</td>
<td>41.67%(25.51%-59.24%)</td>
<td>50%(27.19%-72.80%)</td>
</tr>
<tr>
<td>OXA-48-like</td>
<td>22.2%(10.11%-39.15%)</td>
<td>20%(12.83%-43.66%)</td>
</tr>
<tr>
<td>SHV-like/TEM-like</td>
<td>30.56%(16.34%-48.10%)</td>
<td>50%(27.19%-72.80%)</td>
</tr>
<tr>
<td>CTX-M-G9/CTX-M-G1</td>
<td>16.67%(6.37%-32.81%)</td>
<td>40%(19.11%-63.94%)</td>
</tr>
<tr>
<td>OXA-48-like/CMY-2-like</td>
<td>13.89%(4.66%-29.49%)</td>
<td>15%(3.20%-37.89%)</td>
</tr>
<tr>
<td>CTX-M-G1/CMY-2-like</td>
<td>19.44%(8.19%-36.02%)</td>
<td>25%(8.65%-49.10%)</td>
</tr>
<tr>
<td>CTX-M-G9/OXA-48-like</td>
<td>22.22%(10.11%-39.15%)</td>
<td>25%(8.65%-49.10%)</td>
</tr>
<tr>
<td>CTX-M-G1/OXA-48-like</td>
<td>11.11%(3.11%-26.06%)</td>
<td>10%(1.23%-31.69%)</td>
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<tr>
<td>CMY-2-like/SHV-12-like</td>
<td>16.67%(6.37%-32.81%)</td>
<td>40%(19.11%-63.94%)</td>
</tr>
</tbody>
</table>

CI: Confidence Intervals

Table 4: Distribution of bla genotypes among the clinical K. pneumoniae and E.coli strains and their respective resistance rates to CTX, CAZ, CF, CFM and IMP.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>E.coli</th>
<th>% Resistance to</th>
<th>K. pneumoniae</th>
<th>% Resistance to</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Genotype</td>
<td>Number of isolates (n=36)</td>
<td>Percentage (%)</td>
<td>Number of isolates (n=20)</td>
</tr>
<tr>
<td></td>
<td>SHV-like</td>
<td>18</td>
<td>50</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>TEM-like</td>
<td>15</td>
<td>41.67</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>CTX-M-G9</td>
<td>21</td>
<td>58.33</td>
<td>100</td>
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<td></td>
<td>CTX-M-G1</td>
<td>21</td>
<td>58.33</td>
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</tr>
<tr>
<td></td>
<td>CMY-2-like</td>
<td>15</td>
<td>41.67</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>OXA-48-like</td>
<td>8</td>
<td>22.2</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>SHV-like/TEM-like/CTX-M-G9/CTX-M-G1</td>
<td>11</td>
<td>30.56</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>CTX-M-G9/CTX-M-G1/OXA-48-like</td>
<td>3</td>
<td>8.33</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>CTX-M-G9/CTX-M-G1/OXA-48-like</td>
<td>15</td>
<td>41.67</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>OXA-48-like/CMY-2-like</td>
<td>5</td>
<td>13.89</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>CTX-M-G1/CMY-2-like</td>
<td>7</td>
<td>19.44</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>CTX-M-G1/CMY-2-like</td>
<td>8</td>
<td>22.2</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>CTX-M-G9/OXA-48-like</td>
<td>4</td>
<td>11.11</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>CTX-M-G1/OXA-48-like</td>
<td>4</td>
<td>11.11</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>CMY-2-like/SHV-12-like</td>
<td>6</td>
<td>16.67</td>
<td>100</td>
</tr>
</tbody>
</table>

CTX: Cefotaxime; CAZ: Ceftazidime; CF: Cefalotin; CFM: Cefixime; IMP: Imipenem

Table 5: Relationship between bla genes and resistance to three or more antibiotics in the 56 clinical strains (by χ² test).

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>Resistance to &gt;3 agents</th>
<th>blaTEM-like</th>
<th>p-value</th>
<th>blaSHV-like</th>
<th>p-value</th>
<th>blaCTX-M1</th>
<th>p-value</th>
<th>blaCTX-M9</th>
<th>p-value</th>
<th>blaCMY-2-like</th>
<th>p-value</th>
<th>blaOXA-48-like</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>29/35</td>
<td>0.543</td>
<td>21/28</td>
<td>0.313</td>
<td>6/22</td>
<td>0.248</td>
<td>28/33</td>
<td>0.3</td>
<td>23/25</td>
<td>9/31</td>
<td>0.049</td>
<td>11/11</td>
<td>0.005</td>
</tr>
<tr>
<td>-</td>
<td>5/21</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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Detection of extended-spectrum beta-lactamase and carbapenemase-producing Enterobacteriaceae in Tunisia

Table 6: Relationship between bla genes and resistance to three or more antibiotics in the 56 clinical strains (by multiple logistic regression analysis).

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>$X^2$</th>
<th>p-value</th>
<th>OR (95% CI)</th>
<th>$X^2$</th>
<th>p-value</th>
<th>OR (95% CI)</th>
<th>$X^2$</th>
<th>p-value</th>
<th>OR (95% CI)</th>
<th>$X^2$</th>
<th>p-value</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resistance to &gt;3 agents</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Discussion

In this study, we characterized ESBL- and carbapenemase-producing E. coli and K. pneumoniae clinical isolates collected from Tunisian community and healthcare facilities patients suffering from UTIs. The presence of different Ambler class A β-lactamase encoding genes has been tested by means of RT-PCR with specific primers for bla\textit{TEM}, bla\textit{SHV}, and bla\textit{TEM} [26]. Other β-lactamase encoding genes belonging to class D (bla\textit{OXA-48}) and class C (bla\textit{CMY-2}) have also been tested as previously described [23,27].

Some previous studies have also affirmed the emerging problem of ESBL-producing E. coli and K. pneumoniae isolates in different Mediterranean countries [28].

In this 3-month survey, the incidence rate of ESBL-producing Enterobacteriaceae in urine samples taken from UTI patients was 28.72%. The antimicrobial susceptibility patterns were determined for all the E. coli and K. pneumoniae urine isolates collected. The ESBL-producing isolates showed higher levels of resistance to all antibiotics compared to the non-ESBL-producing isolates. Indeed, data showed that 20% of ESBL-producing E. coli and K. pneumoniae isolates were imipenem-resistant, due to their co-production with an OXA-48-like carbapenemase. This is unsurprising given that ESBL-producing Enterobacteriaceae strains are frequently associated with co-resistance to other antimicrobial agents such as aminoglycosides and fluoroquinolones [29]. The ESBL-producing isolates showed the highest levels of resistance to ampicillin, ceftazidime, and ciprofloxacin. These findings are in accordance with many previous reports [30]. Moreover, ESBLs producers have been proven to be resistant, not only to beta-lactam agents, but also to other antimicrobial agents such as tetracycline, fluoroquinolones, aminoglycosides, and trimethoprim/sulfamethoxazole [31]. Similar resistance profiles were also reported in the present study. There are so many factors responsible for such an elevated rate of antibiotic resistance, some of which are the massive and improper use of antimicrobial agents by health professionals in hospitals, in addition to the self-prescription by community patients [32].

E. coli and K. pneumoniae ESBL-producing isolates from UTIs are increasingly found worldwide, causing serious problems in many countries. Findings from the present study showed that 64% of E. coli and 35% of K. pneumoniae isolates were ESBL-producing bacteria. These findings are consistent with those of Bazaz, et al. who reported a prevalence of ESBLs in K. pneumoniae and E. coli bacteria as high as 59.2%. Reports on ESBL production rate in Enterobacteriales isolates from different countries also show significant variations [33]. In Japan and USA, the prevalence rate of ESBL production in Enterobacteriaceae was reported to be 40 and 44%, respectively [31].

In this study, CTX-M enzymes were the dominant type of ESBLs. Still, the data shows clearly that bla\textit{CTX-M-G1} was the most frequently detected in E. coli and K. pneumoniae isolates (58.33% and 55%, respectively). As well, the bla\textit{CTX-M-9} was detected with a 55% rate. This pattern of results confirms earlier findings showing that the predominance of CTX-M-15 and CTX-M-27-producing ESBLs from human Enterobacteriales isolates is widely present in Tunisia [34]. In addition, these results correlate fairly well with previous studies showing that bla\textit{CTX-M-15} and bla\textit{CTX-M-14} are the most common genes responsible for mediating extended-spectrum cephalosporin resistance in these isolates. Indeed, Mamlouk, et al. detected the bla\textit{CTX-M-15} enzyme in 30% of Enterobacteriales (35% from E. coli and 27% from K. pneumoniae) collected from different wards of Charles Nicolle Hospital in Tunis [35]. More recently in 2014, Ferjani, et al. reported that 88% of ceftaxime-resistant strains harboring \textit{bla}_{OXA-48} group genes also co-harbored \textit{bla}_{CMY-2} like, \textit{bla}_{CTX-M-15} and/or \textit{bla}_{CTX-M-9} genes (Table 4 and Figure 2). This positive association between \textit{bla}_{OXA-48} and/or \textit{bla}_{CMY-2} gene(s) and the resistance to three or more antibiotics was further confirmed by multiple logistic regression analysis (Table 6).

![Figure 2: Distribution of the six group genes in the 36 E. coli and 20 K. pneumoniae clinical strains.](https://example.com/figure2.png)

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resistant *E. coli* strains, isolated from the urine of patients in a Tunisian hospital, harbored the *bla*<sub>CTX-M-15</sub> gene [36]. These studies confirm the current spread of the CTX-M-15 encoding gene, which encodes the most prevalent β-lactamase detected among ESBLs-positive *K. pneumoniae* and *E. coli* strains in Tunisian hospitals. The increased use of cefotaxime and ceftazidime might have contributed to the emergence of ESBLs, particularly these CTX-M-type enzymes. Several previous studies have also discussed the emerging problem of ESBL-producing *E. coli* and *K. pneumoniae* isolates in different geographic regions, including the Mediterranean basin [15] and North Africa [37]. Results of the present study confirm that these CTX-M-type enzymes are the most dominant ESBL type in clinical *E. coli* and *K. pneumoniae* isolates in Tunisia [38]. Interestingly, a similar pattern of results was found in other countries [39].

According to previous reports, the emergence of CTX-M-15 and CTX-M-27 ESBLs from human *Enterobacteriales* isolates has been increasingly reported in many countries around the world, including Tunisia [39].

Data are consistent with those described in the literature [40]. Few studies on molecular analysis of ESBLs-E from Urinary Tract infections were conducted in Tunisia [40]. Currently, CTX-M enzymes have replaced the traditional ESBL types, such as SHV and TEM enzymes, as the most prevalent ESBL type [8].

Hence, the present study is the first to report on the coexistence of CTX-M-1 and CTX-M-9 enzymes in Tunisian ESBL-positive *E. coli* and *K. pneumoniae* uropathogens isolates.

Similarly, in North Africa, many studies have confirmed the increase of ESBL-producing *Enterobacteriales* from urine [38]. In Morocco, Girlich, et al. reported a high rate of ESBL-producing *Enterobacteriales* at the university hospital [41]. Likewise, in Egypt, the CTX-M-15 encoding gene has been detected in clinical isolates of *E. coli* from Cairo [42].

The isolates of *K. pneumoniae* and *E. coli* are common causes of nosocomial infections such as UTIs. These latter can lead to renal failure, if left undiagnosed or late diagnosed. β-Lactam is one of the most effective drugs in UTI treatment. ESBL bacteria, with inactivation of a wide range of β-lactam drugs, especially cephalosporins, and monobactam, can cause treatment failure and increase healthcare costs. Therefore, the study of gene resistance to β-lactamase is important. It seems that the emergence and spread of these bacteria are due to prolonged hospitalization, increased consumption of β-lactam antibiotics (especially ceftazidime), use of catheters, and experimental treatments against antibiotic-resistant [43].

Interestingly, the present study reported the presence of the *bla*<sub>OXA-48-like</sub> gene in eight isolates of *E. coli* (22.2%) and four isolates of *K. pneumoniae* (20%). In addition, seven imipenem-resistant *E. coli* and four *K. pneumoniae* isolates were found to produce OXA-48-like. Several studies on the emergence of OXA-48-producing *Enterobacteriales* have been carried out in Tunisia.

For example, Ktari, et al. [44] reported the spread of 21 (13.7%) *K. pneumoniae* isolates producing the *bla*<sub>OXA-48-like</sub> encoding-gene in a Tunisian university hospital. In 2012, Saidani, et al. [17] examined 21 ESBLs-producing *Enterobacteriales*, with reduced susceptibilities to carbapenems, and found that 5 out of the 21 isolates investigated were OXA-48-like positive.

Indeed, a current study by Mbaya, et al. [45] reports the first description of *K. pneumoniae* carrying the carbapenemase NDM-1 and OXA-like-1 in the Tunisian community setting and confirms that the NDM-1/OXA-like-1-positive ST147 *K. pneumoniae* clone has become endemic in this country, the strain showed the presence of *bla*<sub>CTX-M-15</sub> and *bla*<sub>NDM</sub> genes together with *bla*<sub>TEM</sub>-like, *bla*<sub>SHV</sub>-like, and *bla*<sub>OXA-1-like</sub> genes. This suggests that NDM-1/OXA-48-like-producing ST147 *K. pneumoniae*, although reported as an emerging clone in Tunisia.

More recently, Charfi, et al. [46] reported that among enterobacterial clinical isolates recovered in the Center of Maternity and Neonatology of Monastir in Tunisia, one tested positive for the OXA-48 gene that co-expressed the *bla*<sub>CTX-M-15</sub> gene.

In this study, 5/56 OXA-48-like isolates additionally expressed theblaCTX-M-1 and blaCTX-M-2 genes. In 8/56 cases, we noted the presence of the combination of two β-lactamasases (OXA-48-like and CMY-2-like), highlighting the common combination of several β-lactamasases in a single isolate. Since oxacillinases produced alone possess weak activity against carbapenems, the co-existence of two or more β-lactamasases has become a common bacterial strategy to increase antimicrobial resistance [46].

These findings are of great concern given the fact that the rapid propagation of multidrug-resistant bacteria, particularly cephalosporin and carbapenem-resistant strains, represents a major therapeutic and epidemiological threat. Therefore, the implementation of strict precautionary measures for infection prevention, in addition to regular surveillance studies, is urgent needed in order to limit the increasing spread of these multidrug-resistant bacteria.

**Conclusion**

In conclusion, this study described the high prevalence of CTX-M and OXA-48-like-producing of *E. coli* and *K. pneumoniae* uropathogenic isolates in Tunisian healthcare facilities. However, our results, demonstrate the correlation between genetic and phenotypic profiles of ESBL-producing members of *Enterobacteriales*. The study highlights an escalating crisis of cephalosporins and carbapenemase resistance in *Enterobacteriales*, causing UTIs.
Author contributions statement

1. Rahma Trabelsi contributed to this investigation and carried out most of the experimental procedures. She actively participated in data acquisition, analysis, and interpretation and wrote the article.

2. Mariem Yengui contributed with her efforts and expertise.

3. Amel Mhaya has provided essential reagents and critical revision of the manuscript for important intellectual content.

4. Ahmed Rebai was responsible for the conception, design, and development of the experiments.

5. Corinne Arpin was responsible for the conception, design, and development of the experiments. All authors contributed to the drafting of the manuscript and gave their final approval to the submitted version.

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References


5. Corinne Arpin was responsible for the conception, design, and development of the experiments.

6. Radhouane Gdoura was responsible for the conception, design, and development of the experiments. All authors contributed to the drafting of the manuscript and gave their final approval to the submitted version.

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