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# Characterization of Cephalosporinases Produced by Clinical Isolates of Enterobacteriacae in North Lebanon

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## Abstract

**Background:** The problem of *Enterobacteriacae* resistance to β-Lactamase drugs is of growing concern in hospitals. Enterobacteria have developed multiple mechanisms of resistance to antibiotics, the main one is the enzymatic resistance mediated by the beta-lactamases. This study aims to characterize the occurrence of cephalosporinases in clinical isolates of *Enterobacteriacae* isolates in North Lebanon. Methods. Twenty two strains of *Enterobacteriacae* producing high level of cephalosporinases have been studied. The antibiotic susceptibility of each strain was tested on Mueller Hinton agar contains cloxacilline (250 mg/L) and by using E-test according the guidelines of the Antibiogram Committee of the French Society for Microbiology. The search for plasmid-mediated cephalosporinases was performed using PCR and primers for plasmid-mediated cephalosporinases genes (CMY-2, DHA-1, ACT-1, ACC-1, FOX-1 and MOX-1).

**Results:** Thirteen positive strains were detected, of these 9 strains produced the plasmid-mediated cephalosporinase (CMY-2) and one strain produced the plasmid-mediated cephalosporinase (DHA-1). The remaining 9 strains were high-level chromosomal cephalosporinase producers since they belong to group-three Enterobacteria. They did neither produce plasmid-mediated cephalosporinase, nor did they have resistance to third generation cephalosporins except for cefepim. Two strains (CMUL *E. coli* 021) and CMUL *E. coli* 255) which were not susceptible for cefepim by E-test produced plasmid-mediated cephalosporinase The sequencing result of these 2 *E.coli* strains did not show any mutation in the promoter that is responsible for high expression level of the chromosomal cephalosporinase. All examined strains producing plasmid-mediated cephalosporinase CMY-2 were analyzed by ERIC-PCR technique. The results showed that two of these strains had the same pattern (C4 and C5) and three others had another pattern (C10, C12 and C13).

**Conclusion:** This study shows the variations of cephalosporinases produced by clinical isolates of *Enterobacteriacae* in North Lebanon.

**Keywords:** Cephalosporinases. Antibiotics resistance. Enterobacteria. Lebanon.

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# Introduction

The evolution of extended-spectrum β-lactamases (ESBLs), particularly CTX-M with high levels of resistance to third generation cephalosporins have been increased recently in *Enterobacteriacae* strains mediated via a plasmidic class of cephalosporinase [1]. Class C beta-lactamases (AmpC) is a group of enzymes widely distributed among enterobacteria. They preferentially inactivate narrow spectrum cephalosporins and, in a lesser extent they inactivate the broad-spectrum cephalosporins such as ceftazidime and cefotaxime. However, cefepim is a poor substrate for AmpC in vitro, and it remains active against isolates of enterobacteria that hyper producer of AmpC. Plasmidic cephalosporinase that derived from chromosomal AmpC of different Gram-negative bacteria has emerged since the 1980s. These plasmid-mediated enzymes can be divided into five or six clusters; the *C. freundii* group with LAT types and certain CMY types, the Enterobacter group with MIR-1 and ACT-1, the *M. morganii* group with DHA-1 and DHA-2, the *H. alvei* group represented by ACC-1, and the *Aeromonas* group with MOX-1, FOX-1 and other CMY-type enzymes [2]. AmpC enzymes typically have molecular masses of 34 to 40 kDa and isoelectric points of 8.0, although the isoelectric points of plasmid-mediated FOX enzymes are lower (6.7 to 7.2) [2], and the isoelectric point of an AmpC enzyme of *Morganella morganii*is equal to 6.6 [3]. These enzymes are located in the bacterial periplasmic cell part with the exception of the AmpC β-lactamase of the *Psychrobacter immobilis*, which is secreted mainly into the external medium [4]. They are poorly inhibited by p-chloromercuribenzoate and not inhibited at all by EDTA. Cloxacillin, oxacillin, and aztreonam, however, are good inhibitors [5]. The resistance phenotype of a high level chromosomal cephalosporinase-producing strain is similar to that producing a plasmidic AmpC. There is resistance to first, second and even to third generation cephalosporins, but its susceptible to the fourth generation like cefepim. A striking feature of this development is the global distribution of the strains producing plasmid-determined cephalosporinases. These have been found in Africa (Algeria, Tunisia), Asia (India, Japan, Pakistan, South Korea), Europe (France, Germany, Greece, Italy, Sweden, United Kingdom), the Middle East (Saudi Arabia), North America (United States), and South and Central America (Argentina, Guatemala) [2].

The aim of our study is to characterize the cephalosporinases in twenty two clinical isolates of *Enterobacteriacae* at Nini hospital in North Lebanon.

# Materials and Methods

## Clinical isolates

Twenty-two clinical isolates of *Enterobacteriacae* producing cephalosporinases were obtained from the microbiological laboratory of Nini hospital- North Lebanon. These were isolated between 2009 and 2012 and kept at -80°C in the microbiological department of Azm center. The isolates were obtained from a range of clinical specimens. The most common origin of theses isolates was urine (43%), followed by tracheal aspirations (24.42%), wound (10.86%), perineum, stools (5.43%). All isolates were initially identified with the API 20 E system (biomérieux, Marcy l'Etoile, France) or RapIDone system (Remel, USA), then by the use of mass spectrometry technique (Bruker Daltonik MALDI Biotyper). Susceptibility of the strains was tested using the diffusion disc method [6] and Mueller Hinton agar (Bio-Rad – France) containing cloxacilline (250 mg/L) to check the inhibition of cephalosporinase by cloxacillin according to the recommendations of the Antibiogramm Committee of the French Society for Microbiology (CA-SFM) [7]. The following antibiotics discs (Bio-Rad-France) were tested: Ampicillin (AM) 10 µg, Ticarcillin (TIC) 75 µg, Piperacillin (PIP) 30 µg, Cefoxitin (FOX) 30 µg, Cefotaxime (CTX) 30 µg, Amoxicillin / Clavulanic acid (AMC) 20 µg/10 µg, Ceftazidime (CAZ) 30 µg, Colistin (CS) 50 µg, Ofloxacin (OFX) 5 µg, Piperacillin/Tazobactam (TZP) 75 µg, Trimethroprim/Sulfametoxazole (SXT) 1.25 µg/32.75 µg, Gentamycin (GM) 10 µg, Tobramycin (TM) 10 µg, Netilmicin (NET) 30 µg, Amikacin (AN) 30 µg, Cefepime (FEP) 30 µg, Azrteonam (ATM) 30 µg, Tigecycline (TGC) 15 µg, Moxalactam (MOX) 30 µg, Ertapenem (ETP) 10 µg, Imipenem (IPM) 10 µg and Ticarcillin/Clavulanic Acid (TCC) 75 µg/10 µg. The minimum inhibitory concentration against the following drugs: Cefepime, cefotaxime, ceftazidime and ertapenem, was determined using E-test system on Mueller Hinton agar (Bio-Rad). Breakpoints were interpreted according to the CA-SFM [7]. The strains of *E. coli* or those that belong to group-3 *enterobacteria* which showed resistance to cefepime were analyzed and a sequencing of their AmpC coding gene was performed for potential detection of ESAC (extended spectrum AmpC).

## Bacterial DNA extraction

The DNAs of all *Enterobacteriacae* isolates were extracted automatically using the automate NucliSENS easyMAG according to the manufacturer's instructions and they were stored at - 18° C.

**Table 1.** Primers used for plasmid-mediated AmpC gene amplification and the sequencing primers used in this study.

| Primers | Sequence (5'→ 3') | Reference |
|---|---|---|
| AmpC HN – F<br>AmpC B2 - R | AAAAGCGGAGAAAAGGTCCG<br>TTCCTGATGATCGTTCTGCC | 8 |
| CIT MF<br>CIT MR | TGGCCAGAACTGACAGGCAAA<br>TTTCTCCTGAACGTGGCTGGC | 9 |
| DHA-1-A (F)<br>DHA-1-B (R) | ATGAAAAAATCGTTATCTGC<br>TTATTCCAGTGCACTCAAAATAGC | 8 |
| ACT-1-A (F)<br>ACT-1-B (R) | GGATGAGGTCAAGGATAACG<br>GGTGGATTCACTTCTCTCGC | 12 |
| ACC-1-A (F)<br>ACC-1-B (R) | ATGCAGAACACATTGAAGC<br>CTACTTATTCCCTTCCAATGAGC | 8 |
| FOX1-1-A (F)<br>FOX1-1-B (R) | ATGAATGCCAATTTCATTCACC<br>TCACTCGGCCAACTGACTCAGG | 8 |
| MOXMF<br>MOXMR | GCTGCTCAAGGAGCACAGGAT<br>CACATTGACATAGGTGTGGTGC | 9 |
| ERIC2 | AAGTAAGTGACTGGGGTGAGCG | 10 |

**Table 2.** Positive control strains used in this study.

| Strains | Gene | Reference |
|---|---|---|
| E. coli TOP10 (pCMY-2) | CMY-2 | 8 |
| E. coli TOP10 (pACT-1) | ACT-1 | 8 |
| E. coli TOP10 (pFOX-1) | FOX-1 | 8 |
| E. coli TOP10 (pDHA-1) | DHA-1 | 8 |
| E. coli TOP10 (pACC-1) | ACC-1 | 8 |
| E. coli JM101 (pLRB01) | MOX-1 | 11 |

## PCR of extracted DNA

The extracted DNAs were used as templates to amplify plasmid born AmpC genes (DHA-1, CMY-2, ACT-1, ACC-1, FOX-1 and MOX-1) under the following PCR conditions: denaturation for 5 min at 94°C; 35 cycles of 1 min at 94°C, 1 min at 55°C and 1 min at 72°C; and a final extension step of 10 min at 72°C [8, 9]. The set of primers, which were used to amplify the bla-AmpC genes are presented in **table 1.** Positive control strains used are listed in **table 2.**

Primers AmpC HN-F and AmpC $B_2$-R were used to amplify the chromosomal AmpC gene under the following PCR conditions: denaturationby 1 cycle of 5 min at 94°C; 30 cycles of 1 min at 94°C, 1 min at 55°C and 2 min at 72°C; and a final extension step of 10 min at 72°C.

PCR amplification was performed in a 50 µL mixture containing 5 µL of 10X buffer (Sigma-Aldrich, St Louis, MO, USA), 3µL of $MgCl_2$, 8 µL of mix deoxynucleotide triphosphates (dNTPs; Sigma Aldrich), 0.2 µL of Taq Polymerase (Sigma Aldrich), 2.5 µL of each primer and 2 µL of the DNA template [8, 9]. Amplification of the target regions was performed in a thermal cycler (Veriti thermal cycler, Applied Biosystems), then a migration on gel electrophoresis was performed, and the sizes of plasmid-mediated AmpC genes were revealed by the molecular weight marker (Smart Ladder).

## Sequencing of chromosomal AmpC gene

The PCR products obtained by using the primers of the chromosomal AmpC gene (AmpC HN-F and AmpC B2-R) were used for sequencing. Before sequencing, the PCR products have undergone purification using the purification kit Qiagen QiaQuick according to the manufacturer's instructions. Following the purification, the sequencing of the chromosomal AmpC gene was performed using the sequencing kit Coffet Big Dye terminator cycle sequencing kit (Applied Biosystems®). For each PCR product, two sequencing reactions were performed, in each sample one type of primer was used (forward and reverse), with a total volume of 20 µL. Each reaction contained 4 µL of MixRR (Sigma Aldrich), 2 µL of Buffer 5X, 7µL of purified water, 3 µL of purified PCR product and 4 µL of primer 1 µM (the forward primer in a reaction and the reverse primer in the second), then the thermalcycle (Veritithermacycler, Applied Biosystems®) was run for 30 cycles consisting of initial heat activation at 96°C for 3 min, denaturation at 96°C for 30s, annealing at 55°C

for 15s and elongation at 60°C for 4 min, with a final step at 10°C for 7 min. After the sequencing, purification was performed using the kit Qiagen DyeEx spin kit according to the manufacturer's instructions (Applied Biosystems®), followed by a spectral analysis with genetic analyzer ABI3100.

### Molecular typing

All strains that produced the same type of plasmid-mediated AmpC were typed using the ERIC-PCR technique (Enterobacterial repetitive Intergenic consensus) as described previously [13, 14]. The primer used in this typing technique is listed in **table 1.**

## Results

Among the twenty two strains studied, nine strains produced high level chromosomal AmpC, followed by 12 strains produced the plasmid-mediated AmpC CMY-2 and one strain produced the plasmid-mediated AmpC DHA-1. There were no strains producing one of the following plasmid-mediated AmpC: ACT-1, ACC-1, FOX-1 or MOX-1. The result of susceptibility testing against different antimicrobial agents is shown in **table 3.**

**Table 3.** Mean of antibiotic susceptibility patterns of *Enterobacteriacae* strains against different antibiotics.

| Antibiotics | % S | % R | % I |
|---|---|---|---|
| AM | 0 | 100 | 0 |
| TIC | 0 | 100 | 0 |
| PIP | 0 | 100 | 0 |
| AMC | 0 | 100 | 0 |
| TCC | 0 | 100 | 0 |
| CF | 0 | 100 | 0 |
| FOX | 0 | 100 | 0 |
| CTX | 4.5 | 86 | 9.5 |
| CAZ | 4.5 | 91 | 4.5 |
| FEP | 86.5 | 13.5 | 0 |
| CS | 100 | 0 | 0 |
| OFX | 41 | 50 | 9 |
| TZP | 45.5 | 9 | 45.5 |
| SXT | 41 | 59 | 0 |
| GM | 72.7 | 27.3 | 0 |
| TM | 59 | 36.5 | 4.5 |
| NET | 77.3 | 13.7 | 9 |
| AN | 100 | 0 | 0 |
| ATM | 4.5 | 45.5 | 50 |
| TGC | 77.3 | 0 | 22.7 |
| MOX | 50 | 50 | 0 |
| ETP | 59 | 18 | 23 |
| IPM | 95.5 | 0 | 4.5 |

S: Susceptible I: Intermediate, R: Resistant. MICs of all *Enterobacteriacae* isolates strains against cefotaxime, ceftazidime, cefepime and ertapenem are shown in **table 4.**

All strains of *E. coli* which belong to enterobacteria group-3 and which showed resistance to cefepime through the use of E-test, were analyzed and a sequencing of their AmpC gene was performed for potential detection of ESAC. These strains were CMUL *E. coli* 021 and CMUL *E. coli* 255. The result of their AmpC gene is shown in **figure 1.** There wasn't any mutation at the promoter in the -35 and -10 boxes,neither was there a mutation at position -42 (C to T substitution) that are normally responsible for the appearance of a strong promoter at the origin of the high level expression of *E. coli* chromosomal AmpC. The twelve strains that produced the plasmidic AmpC CMY-2 were analyzed by the ERIC-PCR technique (**fig. 2**). The twelve isolates showed nine distinct fingerprints. Enterobacteria group-1 included the following strains with identical patterns (C10: CMUL *E. coli* 224, C12: CMUL*E. coli* 257 and C13: CMUL*E. coli* 256was the largest group). Group-2 comprised two strains with similar patterns (C4: CMUL *E. coli* 226 and C9: CMUL *E. coli* 225). As for the remainder of the isolates, each produced unique patterns (C2: CMUL *E. coli* 258, C3: CMUL *E. coli* 255, C5: CMUL *E. coli* 021, C6: CMUL *E. coli* 245, C14: CMUL *E. coli* 243, C16: CMUL *E. coli* 250 and C18: CMUL *E. coli* 241)

## Discussion

To our best knowledge, this is the first study in Lebanon, which describes characterization of cephalosporinases produced by clinical isolates of *Enterobacteriacae*.

The results of our study showed that most *Enterobacteriacae* isolates (41%) produced either high level chromosomal AmpC producers or produced plasmid-mediated AmpC CMY-2 (54.5%). Regarding the strains producing plasmid-mediated AmpC, twelve strains (92%) produced AmpC CMY-2 and one strain (8%) produced AmpC DHA. These percentages are consistent with international ones where the major clus-

**Table 4.** MIC determination of the *Enterobacteriacae* strains against cefotaxime, ceftazidime, cefepime and ertapenem.

| Bacterial Strains | MIC on Mueller Hinton agar ( µg/mL) | | | | MIC on Mueller Hinton agar + Cloxacilline (µg/mL) | | | |
|---|---|---|---|---|---|---|---|---|
| | CT (1-2) | TZ (1-4) | PM (1-4) | ETP (0.5-1) | CT (1-2) | TZ (1-4) | PM (1-4) | ETP (0.5-1) |
| CMUL E. coli 021 | >32.00 | >256.00 | 6.00 | 0.38 | >32.00 | 8.00 | 2.00 | 0.125 |
| CMUL C. braakii 006 | 1.50 | 4.00 | 0.64 | 0.125 | 0.125 | 0.19 | 0.047 | 0.032 |
| CMUL E. coli 245 | 12.00 | 8.00 | 0.25 | 0.064 | 0.125 | 0.125 | 0.023 | 0.006 |
| CMUL K. pneumoniae 029 | 1.00 | 8.00 | 0.25 | 1.00 | 0.125 | 0.50 | 0.19 | 0.032 |
| CMUL C. freundii 012 | >32.00 | >256.00 | 1.00 | 0.50 | 3.00 | 12.00 | 0.25 | 0.016 |
| CMUL E. coli 096 | 2.00 | 1.00 | 0.04 | 0.064 | 0.047 | 0.047 | 0.016 | 0.008 |
| CMUL E. coli 255 | >32.00 | 64.00 | 1.50 | 0.19 | 0.50 | 0.75 | 0.125 | 0.016 |
| CMUL E. coli 221 | >32.00 | 24.00 | 0.38 | 0.19 | 0.28 | 0.38 | 0.032 | 0.012 |
| CMUL E. cloacae 012 | >32.00 | 64.00 | 0.50 | 0.19 | 3.00 | 2.00 | 0.047 | 0.032 |
| CMUL E. coli 258 | >32.00 | 32.00 | 0.38 | 0.064 | 1.00 | 2.00 | 0.125 | 0.012 |
| CMUL C. freundii 009 | >32.00 | 12.00 | 0.09 | 0.19 | 0.125 | 0.19 | 0.016 | 0.012 |
| CMUL E. coli 226 | >32.00 | 96.00 | 0.38 | 0.094 | 0.75 | 2.00 | 0.125 | 0.016 |
| CMUL E. coli 243 | >32.00 | 64.00 | 0.38 | 0.064 | 0.50 | 0.75 | 0.125 | 0.016 |
| CMUL E. coli 225 | 24.00 | 24.00 | 0.01 | 0.004 | 0.023 | 0.064 | <0.016 | <0.002 |
| CMUL E. kobei 015 | >32.00 | 16.00 | 0.12 | 0.19 | 0.064 | 0.064 | 0.016 | 0.002 |
| CMUL E. coli 224 | 6.00 | 8.00 | 0.125 | 0.047 | 0.032 | 0.032 | <0.016 | 0.002 |
| CMUL E. cloacae 017 | >32.00 | 64.00 | 1.00 | 0.19 | 0.50 | 0.38 | 0.023 | 0.008 |
| CMUL E. coli 257 | >32.00 | >256.00 | 1.00 | 0.38 | 1.00 | 2.00 | 0.094 | 0.032 |
| CMUL E. coli 256 | >32.00 | 16.00 | 0.38 | 0.125 | 0.094 | 0.125 | 0.023 | 0.004 |
| CMUL E. cloacae 013 | >32.00 | 4.00 | 0.094 | 0.38 | 0.19 | 0.125 | 0.016 | 0.016 |
| CMUL E. coli 250 | >32.00 | 24.00 | 0.19 | 0.064 | 0.032 | 0.047 | <0.016 | 0.002 |
| CMUL E. coli 241 | >32.00 | >256.00 | 0.50 | 0.064 | 0.50 | 1.50 | 0.064 | 0.012 |

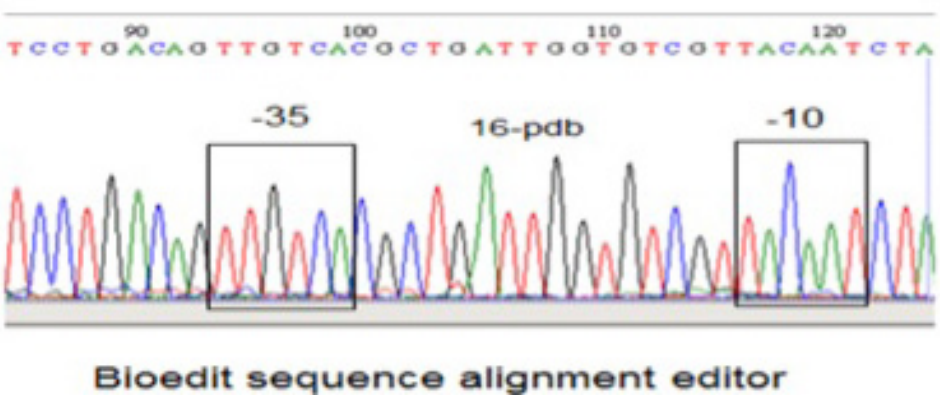


**Figure 1.** Chromatogram showing the sequence of the promoter of the chromosomal AmpC gene.

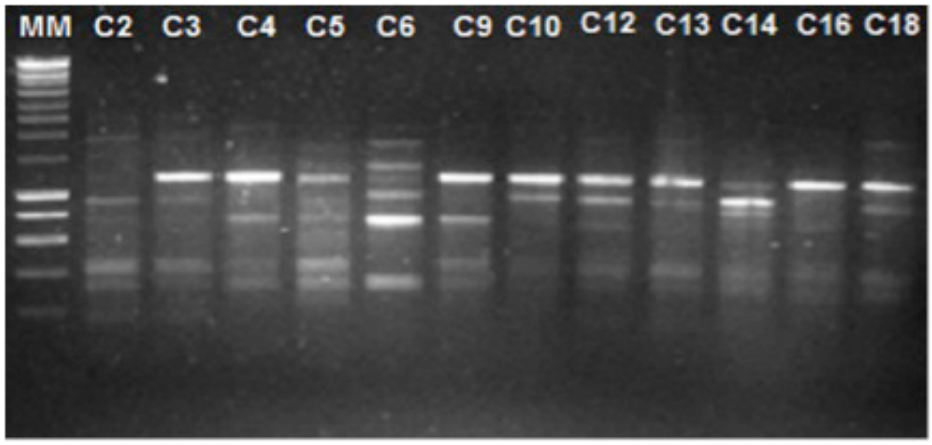


**Figure 2.** Result of the migration of the ERIC-PCR products.

**MM:** Molecular weight marker, **C2:** CMUL E. coli 258, **C3:** CMUL E. coli 255, **C4:** CMUL E. coli 226, **C5:** CMUL E. coli 021, **C6:** CMUL E. coli 245, **C9:** CMUL E. coli 225, C10: CMUL E. coli 224, **C12:** CMUL E. coli 257, **C13:** CMUL E. coli 256, **C14:** CMUL E. coli 243, **C16:** CMUL E. coli 250 and **C18:** CMUL E. coli 241.

ter is the one that gathers the enzymes that are very similar to CMY-2. A study conducted in France at Nantes University Hospital over 5 years, has showed that all examined *E. coli* strains produced the plasmid-mediated AmpC CMY-2 ( add reference ?). Moreover, a study conducted in China [15] and designed to determine the prevalence of plasmid-mediated AmpC in strains of *E. coli* and *Klebsiella spp*., showed that among 1935 clinical isolates of *E. coli*, *K. pneumoniae* and *oxytoca*, 54 strains (2.79%) produced a plasmid-mediated AmpC, 41 strains produced AmpC DHA and 13 strains were produced AmpC CMY-2. A similar study conducted in Japan [16] showed that among 46 strains of *k. pneumoniae* resistant to cephalosporins, only two strains produced the plasmid-mediated AmpC CMY-2 and 41 produced the plasmid-mediatedAmpC DHA-1.The antibiogram of our strains showed that all strains were resistant to cefalotin and cefoxitin, while 86% of the strains were resistant to cefotaxim and 91% to ceftazidime. However, 86.5% of the strains were susceptible to cefepim and only 3 strains were resistant to? The determination of MIC showed that 2 strains out of 3 were not susceptible to cefepim (CMUL *E. coli* 021 with a MIC = 6 µg/mL and CMUL *E. coli* 255 with a MIC = 1.5 µg/mL). Sequencing of their chromosomal AmpC encoding genes did not show any mutation in the promoter in the -35 and -10 box or a mutation in the nucleotide at position -42 (C to T substitution) that are normally responsible for the appearance of a strong promoter that causes the high level expression of the chromosomal AmpC in *E.. coli*. Therefore, resistance to cefepim in these 2 strains may be due to a membrane impermeability since the 2 strains were also resistant to moxalactam which is very sensitive to the membrane impermeability [17] and both strains produce plasmid-mediated AmpC CMY-2. Susceptibility testing on Mueller-Hinton medium with cloxacillin usimg E-test showed a total recovery of susceptible strains to cephalosporins, however, for CMUL *E. coli* 021, there was a partial demonstration of susceptibility to cephalosporins on Mueller-Hinton medium with cloxacillin and there was no reaction of synergy observed between clavulanic acid/amoxicillin and third-generation cephalosporins. Therefore, other beta-lactamase (ESBL) secreted by this strain may explain the increase cefotaxime MIC on Mueller Hinton with cloxacillin. The synergy reaction was absent on Mueller-Hinton medium with cloxacillin, but this may be due to the outer membrane impermeability since the strain was resistance to moxalactam and it will be difficult to demonstrate this synergy in case of impermeability. The molecular typing by the ERIC-PCR technique showed the presence of two distinct clusters. First cluster of two strains and three strains, while the other strains each one gave a specific pattern. It is highly desirable to conduct further studies using the technique of pulsed field gel electrophoresis (PFGE) to figure out whether the strains within the same cluster were belonged actually to the same clone or not. In conclusion, this work presents characterization of cephalosporinases produced by clinical isolates of *Enterobacteriacae* in North Lebanon, and demonstrates the different plasmid-mediated types of the cephalosporinases.

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