ISSN 2449-8955 European Journal of Biological Research *Research Article*

**DOI:** http://dx.doi.org/10.5281/zenodo.3776651

# The first report of the coproduction of CMY-16 and ArmA 16S rRNA methylases in carbapenemase-ESBL producing *Escherichia coli* isolates

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**Received:** 18 March 2020; **Revised submission:** 20 April 2020; **Accepted:** 27 April 2020

**http://www.journals.tmkarpinski.com/index.php/ejbr**



**ABSTRACT:** The main aim of this work was to assess the occurrence and to characterize AmpC genes and to investigate the co-existence of 16S rRNA methylases and carbapenemases genes among the ESBL producing *Escherichia coli* strains. 180 *Escherichia coli* clinical strains were collected from the university hospital of Constantine located in the eastern part of Algeria. 42 ESBL-producers were phenotypically identified and also confirmed genotypically able to produce *CTX-M-15* [n=33], *CTX-M-1* [n=5], *CTX-M-14* [n=1], *SHV-2* [n=1], and two strains have been revealed producing the $bla_{\text{OXA-48}}$ genes associated with $bla_{\text{TEM-1}}$. Among the ESBL-producing strains three expressed additionally an AmpC phenotype which corresponded to the carriage of a $bla_{\text{CMY}}$ gene shown by sequencing to correspond to *CMY-2* (1 isolate) *CMY-16* (2 isolates). The two *E. coli* isolates produce *CMY-16* that belonged to phylogroup D while the single *CMY-2* producing isolate belonged to phylogroup C. Antibiotic resistance of the aminoglycoside family by production of 16S rRNA methylases was detected by an end-point multiplex PCR assay which concerns genes coding for different 16S rRNA methylases (*rmtD*, *rmtA*, *rmtB*, *armA*, *npmA*, and *rmtC*). An *armA* gene was identified in 2 strains. This study shows for the first time the co-existance of *CMY-16* and *armA* genes with $bla_{\text{TEM-1}}$ and $bla_{\text{OXA-48}}$ producing *E. coli* strains.

**Keywords:** AmpC β-lactamase *CMY-16*; *Escherichia coli*; ArmA 16S rRNA methylases; ESBL coproduction.

## 1. INTRODUCTION

The worldwide spread of genes conferring resistance to different antibiotics is considered as a major cause of mortality in hospitals [1]. *Escherichia coli* strains have been characterized by their resistance to antibiotics used in therapy [2]. The emergence of resistant strains to different families of antibiotics such as β-lactams, and aminoglycosides pose a serious therapeutic problem in hospitals [3, 4]. In addition to the spread of β-lactam resistance, *E. coli* sequence type (ST131) has disseminated internationally and the strains

are considered to be truly pathogenic due to the spectrum of infectious they cause in both communities and hospitals [5].

The resistance of *E. coli* strains to β-lactam antibiotics is rapidly disseminated and it is mainly related to the production of β-lactamases [6]. The production of AmpC is considered to be one of the mechanisms of resistance to β-lactam in the *Enterobacteriaceae* family, conferring resistance to all β-lactam antibiotics except fourth-generation cephalosporins and carbapenems [7]. The genes that code for these enzymes are of chromosome or plasmid origin [8]. The AmpC-lactamases were identified among *Enterobacteriaceae*, particularly in *Escherichia coli*, *Salmonella enterica*, *Klebsiella pneumonia* and *Proteus mirabilis* and also in naturally AmpC-producing species [9, 10]. The dissemination of resistance to aminoglycosides was also identified [11]. Aminoglycoside resistance by the production of 16S rRNA methylases is the most recognized type of resistance to these antibiotics [3, 12]. The first discovery of the *armA* gene occurred in 2003. Then, after the different studies, eight plasmid-mediated 16S rRNA methylases (*rmtC*, *rmtA*, *armA*, *rmtE*, *rmtB*, *rmtD*, *npmA*, and *rmtF*) were detected in clinical strains of gram negative bacilli [13, 14]. Currently, eleven 16S rRNA methylases genes (*armA*, *rmtB*, *rmtD*, *rmtE*, *rmtC*, *rmtF*, *rmtA*, *npmA*, *rmtD2*, *rmtG and rmtH*) have been identified, of which *ArmA* and *RmtB* were the most frequently found methylase in strains of *Enterobacteriaceae* [15, 16]. 16S rRNA methyltransferase enzymes are generally seen together with carbapenemases and extended-spectrum beta-lactamase (ESBL). Coproduction of β-lactamase and 16S rRNA methylases among strains of *Enterobacteriaceae* leads to resistance to all treatment modalities [11, 16].

In this work we aimed to characterize the AmpC β-lactamase and 16S rRNA methylases among the ESBL and carbapenemases producing *Escherichia coli*.

## 2. MATERIALS AND METHODS

### 2.1. Collection of strains

180 *E. coli* strains were collected from patients hospitalized at the university hospital of Constantine Abdelhamid Ben Badis, Algeria. These samples were collected from different pathological humans' specimens including wounds, pus, urine, blood and other body fluids such as gastric fluid and ascites fluid. These strains were characterized by standard bacteriological technique and the classic biochemical gallery and confirmed at the species level by the technique of MALDI-TOF MS (Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry) by using Microflex LT (Bruker Daltonics, Germany) based on the MALDI BioTyper database (version IVD 2.2 DB-5989 MSP).

### 2.2. In vitro antimicrobial sensitivity and phenotypic identification of resistance mechanisms

Antibiotic sensitivity testing was performed by the disk diffusion method on Mueller-Hinton agar and Microscan according to CLSI 2012 guidelines, for the following antibiotics: beta-lactam family (aztreonam, amoxicillin/clavulanate, ampicillin, cefotaxime, cefuroxime, ceftazidime, cefepime, piperacillin/tazobactam, temocillin, cefoxitin, ertapenem, meropenem), sulfonamides family (cotrimoxazole), quinolone family (ciprofloxacin), aminoglycoside family (amikacin and gentamicin).

All cefoxitin-resistant isolates (diameter of the inhibition zone <18 mm according to CLSI 2012) from this collection were selected for screening for AmpC genes. Extended-spectrum β-lactamase production has been detected by studying the resistance of the strains to third-generation cephalosporins and also by the double-disk synergy test.

Strains with reduced sensitivity to meropenem or ertapenem were identified phenotypically for the production of carbapenemase enzymes by Carba NP assay as previously reported by Nordmann et al. [17], the

modified Hodge test and the inhibition of the metallo-β-lactamase activity by ethylenediaminetetraacetic acid (EDTA) as previously described by Bakour et al., and Yong et al. [18, 19]. Aminoglycosides resistance induces the research of 16S rRNA methylases production in the isolates by detecting the genes involved in their biosynthesis.

### 2.3. Extraction of DNA from bacterial strains

By the boiling lysis method, the DNA has been obtained. Suspensions of the analyzed strain and the control were prepared in 200 µL of sterile distilled water, boiled at (100ºC for 10 min and centrifuged at 13'000 × g for 10 min). The supernatants were used as DNA template for PCR amplification assays [20].

### 2.4. Genotypic identification of ESBL, AmpC, 16S rRNA methylase and carbapenemase genes

Screening for genes encoding conventional ESBL (*bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{CTX-M}$), AmpC genes (*CMY, FOX, ACC, MIR, ACT, MOX, DHA*), carbapenemase genes (*bla*$_{VIM}$, *bla*$_{KPC}$, bla$_{IMP,}$ *bla*$_{NDM}$, *bla*$_{OXA-48}$) and the 16S rRNA methylases genes (*armA*, *rmtA*, *rmtB*, *rmtC*, *rmtD*, and *npmA*) was performed by an end-point multiplex PCR assay as previously described by Perez-Perez et al. and Bogaerts et al. [8, 20], followed by sequencing the PCR products such as (*CTX-M, SHV, TEM, CMY*). The products of the sequencing were compared to the sequences reported in Gen-Bank [21].

### 2.5. Sequence type ST131 determination

Identification of the ST131 clone was carried out using O25b-ST131 clone allele-specific PCR for the *papB* gene [22].

### 2.6. Phylogenetic groups

The determination of phylogenetic groups of *E. coli* strains was performed by PCR method for phylo-group assignment according to revised Clermont method. Each strain was assigned to one of eight major phylo-groups that are as follows (A, B1, B2, C, D, E, F, and clade I) [23].

## 3. RESULTS

### 3.1. Bacterial strains and ESBL producers

The double-disk synergy test for phenotypic detection of ESBL production revealed that 42 samples of *E. coli* (23%) produced ESBLs. The strains were mainly recovered from urine samples (60%), pus samples (30%) and those of other body fluids (10%).

Antibiotic resistance profile and sensitivity showed that all ESBL strains expressed resistance to multiple antimicrobial agents. The strains are highly resistant to ampicillin, cefuroxime, cefotaxime, cefepime and aztreonam. The strains resistance to other antibiotics was high for gentamicin (63%) ciprofloxacin (61%) and cotrimoxazole (60%) but low for amikacin (5%). The isolates showed a high sensitivity to piperacillin-tazobactam (95%), ertapenem and meropenem with a frequency of (95%), temocillin (92%), cefoxitin (85%), amoxicillin-clavulanate (83%), and ceftazidime (66%) (Table 1).

According to CLSI 2012 recommendations, two isolates were obtained from two different patients urine samples were resistant to ertapenem (diameter of the inhibition zone $\leq$ 18 mm) and to the majority of antibiotics used. However, these two strains exhibited intermediate resistance to meropenem and they were sensitivity to ciprofloxacin and cefepime.

**Table 1.** The antibiotic resistance and sensitivity profile of the ESBL strains of *E. coli*.

| Antibiotics | ESBL strains of *E coli* | |
|---|---|---|
| | Phenotype | Percentage % |
| Ampicillin | Resistant (R) | 100 |
| Cefuroxime | | 99 |
| Cefotaxime | | 99 |
| Cefepime | | 70 |
| Aztreonam | | 65 |
| Gentamicin | | 63 |
| Ciprofloxacin | | 61 |
| Cotrimoxazole | | 60 |
| Amikacin | | 5 |
| Piperacillin-Tazobactam | Sensitive (S) | 95 |
| Ertapenem | | 95 |
| Meropenem | | 95 |
| Temocillin | | 92 |
| Cefoxitin | | 85 |
| Amoxicillin-clavulanate | | 83 |
| Ceftazidime | | 66 |

### 3.2. Detection of carbapenemase production

#### *3.2.1. Carba NP test*

Carba NP is a rapid test for screening carbapenemase producers in *Enterobacteriaceae*. In our study this assay is based on the ertapenem's hydrolysis with a color change of a phenol indicator from red to orange or yellow (positive result) [17]. Only two strains of *E. coli* (*E. coli* 175, *E. coli* 178) were found to be producing carbapenemases (Figure 1).

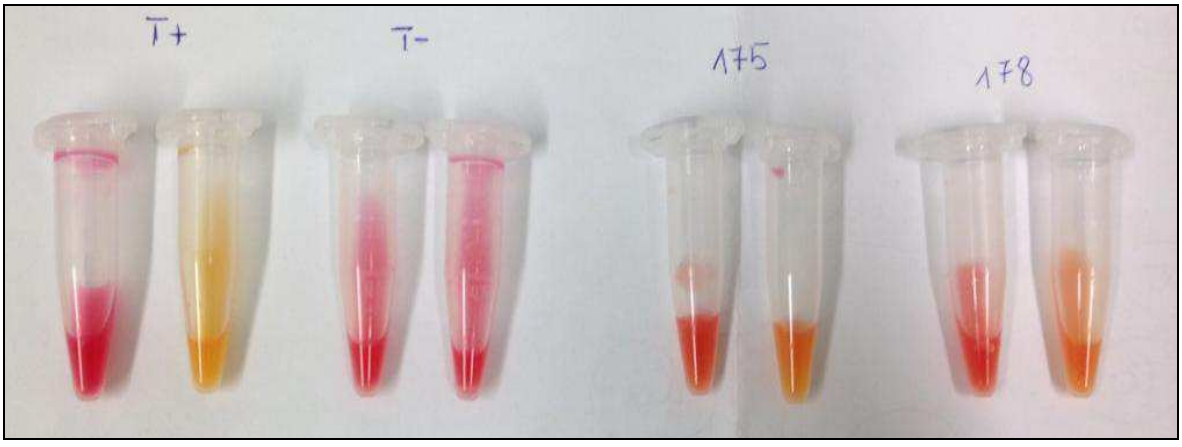


**Figure 1.** Carba NP test positive with two strains of *E. coli* OXA type; hydrolysis of ertapenem with a color change of a phenol indicator from red to orange or Yellow. From left to right the second tube is the one that is inoculated for (T+, T-, 175, 178).

T+: positive control NDM positive *E. coli*; T-: negative control *E. coli* J53

178: *E. coli* 1 (Ec1) carbapenemase positive; 175: *E. coli* 2 (Ec2) carbapenemase positive

### *3.2.2. Modified Hodge test*

The presence of a carbapenemase is exposed by the deformation of the zone of inhibition due to the enzymatic activity around the antibiotic close to the suspect strain (Figure 2). By contrast, the EDTA test was observed negatively because the carbapenemases produced by these two strains of *E. coli* were not liked to NDM, VIM or IMP genes expression.

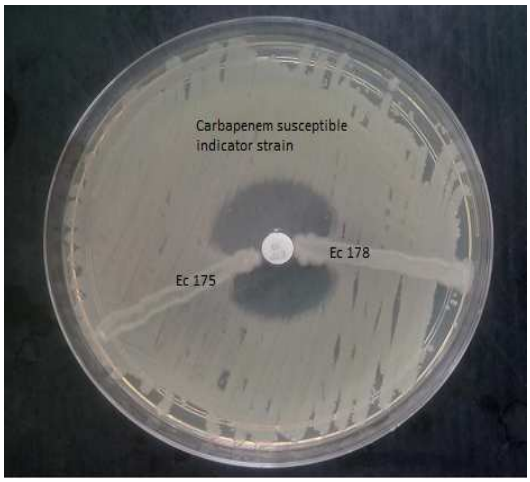


**Figure 2.** Modified Hodge test positive with two strains of *E. coli* (Ec 175 and Ec 178); inactivation of carbapenem by carbapenemase-producing strains that enables a carbapenem-susceptible indicator strain to extend growth towards a carbapenem-containing disc, along the streak of inoculums of the tested suspected carbapenemase.

### 3.3. Genotypic analysis of antibiotic resistance genes

The results of the end-point multiplex PCR and sequencing showed the presence of $bla_{CTX\text{-}M}$ in 39 strains (5 $bla_{CTX\text{-}M\text{-}1}$, 1 $bla_{CTX\text{-}M\text{-}14,}$ 33 $bla_{CTX\text{-}M\text{-}15,}$) while $bla_{SHV\text{-}2}$ was detected in one isolate and $bla_{TEM\text{-}1}$ in two isolates. In addition the detection of AmpC gene like *CMY-2* was showed in one strain of *E. coli* among the CTX-M-15 producing strains and the presence of *CMY-16* in two strains of *E. coli* (TEM-1). These two strains have also been revealed to be producing carbapenemases (OXA-48) and 16S rRNA methylases (ArmA).

### 3.4. Sequence type ST131 and phylogenetic groups determination

Detection of the ST131 clone using PCR targeting the papB gene revealed that 16 isolates (CTX-M-15) were ST131 positive and that they belonged to phylogroup B2. While the other strains were ST131 negative, among them two isolates (CTXM-15) belonged to group B2, two (CTXM-15) to group A, five (CTXM-1) to group B1, eight (CTXM-15) to group D, three (CTXM-15) to group C, two (CTXM-15) to group F, one (CTXM-14) to group D, one (SHV2) to group B1 and two (TEM-1) belonged to group D (Table 2).

**Table 2.** Phylogenetic groups and sequence type ST131 of the ESBLs producing strains.

| **Phylogenetic groups** | **B2** | | **A** | **B1** | | **D** | | **C** | **F** | **D** |
|---|---|---|---|---|---|---|---|---|---|---|
| ESBL type | CTX-M-15 | CTX-M-15 | CTX-M-15 | CTX-M-1 | SHV-2 | CTX-M-15 | CTX-M-14 | CTX-M-15 | CTX-M-15 | TEM-1 |
| ST131 | Pos (+) | Neg (-) | Neg (-) | Neg (-) | Neg (-) | Neg (-) | Neg (-) | Neg (-) | Neg (-) | Neg (-) |
| Number | 16 | 2 | 2 | 5 | 1 | 8 | 1 | 3 | 2 | 2 |

## 4. DISCUSSION

In our study, *E. coli* produced CTX-M15 that is the most common ESBL type, which confirms previous studies [24]. A similar situation is also observed in another country in North Africa in Morocco [25] which confirms the high dissemination of CTX-M15 producing *E. coli* isolates. Comparing to other studies [26], our study confirms the dissemination of *E. coli* ST131 associated with the CTX-M-15 Extended-Spectrum beta-lactamase and attests the serious worldwide problem of multidrug-resistant pathogen *E. coli* strains.

CTX-M1, CTX-M14, TEM-1, SHV-2 and OXA-48 genes were detected in *E. coli* strains identified in this study have been also mentioned in previous findings [25, 27]. Nevertheless, the SHV-2 ESBL that was identified in this study was never described to date among *E. coli* clinical isolates in Algeria, but one study revealed the detection of SHV-12 ESBL in clinical strains of *E. coli* isolated from hospitals in the west of Algeria [27].

The *armA* gene which was detected in our analyzed strains was identified previously in 2016. A report from the west of Algeria [27] had revealed that there were four ArmA producers among *E. coli* strains which were gathered from some hospitals in the western part of Algeria.

Concerning the AmpC β-lactamase production in this study, the sequence analysis, showed that $bla_{CMY}$ genes detected were $bla_{CMY\text{-}2}$ (one isolate) and $bla_{CMY\text{-}16}$ (two isolates). The CMY-2 type is the most frequent, especially in Europe (France, Spain, Italy, and Turkey [28, 29], in Canada, Argentina, Tunisia and Algeria [27, 30, 31]. So, our results are consistent with the previous results.

CMY-16, a variant of the CMY lineage was first detected in *Proteus mirabilis* that was isolated from Italy [32] and since then it has been detected throughout the world. CMY-16 was found to be the most prevalent variant of AmpC β-lactamases in Europe [33]. A study in Italy demonstrated the co-existance of CMY-16 in association with TEM-92 which is an ESBL in *Proteus mirabilis* isolates [34]. Similarily as in another study in Croatia, CMY-16 was found in *Proteus mirabilis* in association with TEM-1 [35]. In Switzerland CMY-16 was detected in association with OXA-48, CTXM-15 and ArmA [36] in *Klebsiella pneumonia* strains. As well as, In Tunisia they have reported the coproduction of CMY-16 and OXA-1 in *E. coli* strains [37].

Here, in our study we report the first detection of CMY-16 gene in association with TEM-1 BLSE, OXA-48 carbapenemase and ArmA 16S rRNA methylases in *E. coli* strains. The combination reported in this study was not described before: two strains of *E. coli* CMY-16 co-produced TEM-1 plus OXA-48 and ArmA. The phylogenetic study showed that these two strains belonged to phylogenetic group D and they were ST131 negative. In fact they were collected from two different patients.

So, the dissemination of CMY-16 and armA genes detected in carbapenemase-ESBL producing *E. coli* clinical samples is a major problem. The emergence of this resistant type should be controlled and limited through molecular surveillance.

## 5. CONCLUSION

According to data published, we describe here the first detection of multiresistant isolates of *E. coli* that co-produced: CMY-16, TEM-1, OXA-48 and ArmA. Our study confirms the major problem of multi-resistance of *E. coli* strains. The association of multiple resistance genes in *E. coli* strains is a disturbing situation. So, there is a need to detect and to control their dissemination by creating new treatments to contain the risk of spread.

**Authors' Contributions:** MM: Collection of samples, designed the study and protocol, Conception of the paper and design of the manuscript; acquisition of data, Analysis and interpretation of data, managed literature search, writing and revision of the manuscript. KB: provided the strains, administrative support. PB and YG: technical support, development of methodology, review of the manuscript. All authors read and approved the final manuscript.

**Conflict of Interest:** The authors have no conflict of interest to declare.

**Funding:** University of Mentouri Brothers, Constantine1, Algeria is acknowledged for funding this work.

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