



ISSN NO. 2320-5407

Journal homepage: <http://www.journalijar.com>
Journal DOI: [10.21474/IJAR01](https://doi.org/10.21474/IJAR01)

**INTERNATIONAL JOURNAL
OF ADVANCED RESEARCH**

RESEARCH ARTICLE

Carbapenem resistance due to BlaOXA-48 Clone ST38 among ESBL-producing *Escherichia coli* isolates from patients hospitalised at the university hospital of Constantine in Algeria.

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Manuscript Info

Manuscript History:

Received: 12 May 2016
Final Accepted: 19 June 2016
Published Online: July 2016

Key words:

Escherichia coli, Carbapenemases, Bacterial resistance, University Hospital of Constantine, Algeria.

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Abstract

The aim of this study was to investigate the prevalence and to assess the frequency and diversity of carbapenemases produced by *Escherichia coli* isolates in the university hospital of Constantine in Algeria. A total of 235 strains of *Escherichia coli* were isolated from patients from February 2014 until June 2015 and identified with biochemical approaches and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF). Antimicrobial susceptibility was determined using the disk diffusion and E-test methods. Carbapenemase activity was studied using modified Hodge test, modified Carba NP test, and EDTA test. Carbapenem resistance determinants were detected by real-time PCR and standard polymerase chain reaction (PCR) followed by sequencing. The genes coding for the ESBL were determined by PCR and sequencing. Carbapenemase-producing *Escherichia coli* isolates were typed by MLST and their transferability was studied by a conjugation experiment followed by antibiogram and PCR amplification of the plasmid DNA. As results, two strains of *E. Coli* were resistant to ertapenem with MIC= 4 mg/l. They presented a resistance to beta-lactams, to aminoglycosides and fluoroquinolones. However, they were susceptible to imipenem and colistin. In addition, they were revealed producing the blaTEM-1 gene. They were positive with the modified Hodge test and modified Carba NP test. Real-time PCR, standard PCR and sequencing showed the presence of blaOXA-48 gene in these strains. The results of conjugation experiment and the extraction of the plasmid which were negative showed that these resistance genes are chromosomally encoded. MLST with seven housekeeping genes demonstrated that the two strains of *E. Coli* OXA-48 producers belong to ST38. In conclusion, here, we demonstrate the emergence and dissemination of carbapenemase-producing *E. Coli* strains at the university hospital of Constantine in Algeria and we report the first blaOXA-48 ST38 producing *E. Coli* clinical isolates from patients in Algeria.

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Introduction:

Escherichia coli is the most common agents among clinical isolates causing infection in humans. The dissemination of multidrug-resistant *Escherichia coli* strains is considered a major cause of morbidity and mortality in hospitals [1]. Along with the massive use of antibiotics including β -lactam antibiotics, bacterial β -lactamases have evolved towards diversification, expanding their spectrum of activity and their dissemination among many species of Enterobacteriaceae [2]. The emergence of resistance to carbapenems poses a serious therapeutic problem in hospitals because carbapenems are often antibiotics used for the treatment of infections caused by multidrug-resistant Gram-negative bacteria [3, 4].

Different types of carbapenemases (A, B and D) have been described, extensively worldwide in Enterobacteriaceae [5]. Among these enzymes, OXA-48 had first been reported and identified from a clinical *Klebsiella pneumoniae* isolate in Istanbul, Turkey, in 2001[6]. The gene *bla_{oxa-48}*, was also identified in *Escherichia coli* and *Citrobacter freundii*, still in Turkey [7]. Since 2008, this gene has been described in many other countries, most often in *K. Pneumoniae* isolates [8, 9, 9, 10]. OXA-48 is one of the latest reported carbapenemases and has been identified mostly from Mediterranean countries [11].

In this report we aimed to study the emergence and dissemination and to assess diversity of carbapenemases produced by *Escherichia coli* isolates from patients hospitalised in the university hospital of Constantine (Algeria). In this report, we describe the first detection of *bla_{OXA-48}* ST 38 producing *E. Coli* clinical isolates in Algeria.

Materials and methods:

Bacterial isolates:

A total of 235 clinical isolates of *E. Coli* were obtained from hospitalized and nonhospitalized patients at the University Hospital of Constantine, Algeria. These strains come from different pathological specimens, and were collected from February 2014 until June 2015. The isolates were first identified with standard bacteriological technique and biochemical approaches using the API system 20E® (biomérieux., Marcy l'Etoile, France) and confirmed by the Bruker Daltonics Microflex matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometer (Bremen, Germany), as previously described [12].

Antimicrobial susceptibility:

Antibiotic susceptibility was determined by the disk diffusion assay on Mueller-Hinton agar according to recommendations of the Antibiotic Committee of the French Society for Microbiology (<http://www.sfm-microbiologie.org>). Sixteen antibiotics were tested, including amoxicillin, amoxicillin + clavulanate, ticarcillin, piperacillin, cefazolin, cefoxitin, cefotaxim, aztreonam, ertapenem, imipenem, gentamycin, amikacin, nalidixic acid, ciprofloxacin, sulfamethoxazole + trimethoprim and colistin.

The susceptibility to carbapenems (ertapenem) was tested with using The Etest (biomérieux). The production of extended-spectrum β -lactamase (ESBL) was screened by the double-disk synergy test [13, 14].

Detection of carbapenemases :

Carbapenemase activity was determined using the modified Carba NP test, the modified Hodge test [15] and the inhibition of the metallo- β -lactamase activity by ethylenediaminetetraacetic acid (EDTA) as previously published [16].

Molecular analysis:

Resistance mechanisms:

The screening of the carbapenemase genes *bla_{VIM}*, *bla_{KPC}*, *bla_{NDM}*, *bla_{OXA-48}* was carried out by real-time PCR and standard polymerase chain reaction followed by sequencing [17, 18]. The genes coding for the *esbls* (*blatem*, *blashv* and *blactx-M*) were detected by polymerase chain reaction (PCR) using specific primers and determined by sequencing the PCR products [19, 20].

Molecular epidemiology:

Sequence type (ST) determination:

Multilocus sequence typing (MLST) of the isolates of *E. Coli* was carried out using seven conserved housekeeping genes (*adh*, *fumc*, *gyrb*, *icd*, *mdh*, *pura* and *reca*), as reported at the *E. Coli* MLST Database (<http://mlst.warwick.ac.uk/mlst/dbs/Ecoli>). The fragments of the seven housekeeping genes were amplified and sequenced as previously described on the following website <http://mlst.ucc.ie/mlst/dbs/Ecoli>. A different allele number was attributed to each distinct sequence within a locus, and a ST number was given to each distinct combination of alleles.

Transferability of antibiotic resistance genes:

The transferability of the resistance phenotype was studied by a conjugation experiment between our clinical donor isolates and azide-resistant *E. Coli* strain J53 as a recipient. The transconjugants were selected on Mueller-Hinton agar plates containing 2 μ g/ml ertapenem and 100 μ g/ml sodium azide, as described previously [21]. Then, the results of the conjugation experiment were confirmed by an extraction of the plasmid.

Results:

Antibiotic susceptibility:

In our study the strains of *E. Coli* were mainly isolated from urine samples with a frequency of 59.57 % followed by pus samples and those of gastric fluid with respectively 34.04 % and 6.38 %.

The results of resistance rate of 235 *Escherichia coli* strains to antibiotics testing are shown in Table1. They revealed that the isolates showed resistance to multiple antimicrobial agents. The strains showed a high resistance to amoxicillin, ticarcillin, amoxicillin+clavulanate, cefazolin, sulfamethoxazole+trimethoprim, piperacillin and nalidixic acid. By contrast, two antibiotics were active against all isolates studied; 235 (100%) of the 235 isolates were susceptible to colistin, and 235 (100%) to imipenem. However, according to the CASFM 2015, two strains (0.85%) of the 235 isolates were resistant to ertapenem and they were found resistant to almost all antibiotics tested, including beta-lactams, aminoglycosides, and fluoroquinolones (table2). So, these two strains of *E. Coli* were found to be ESBL and carbapenemases producers, they were isolated from the urine sample from two different patients at the university hospital of Constantine, Algeria.

Phenotypic detection of carbapenemases:

In our study the Carbapenemase production was detected phenotypically using the modified Carba NP test, the modified Hodge test and the disk approximation tests using EDTA. Only two isolates of *E. Coli* were positive for production of carbapenemase, with Carba NP test and Hodge test (figure1, 2). With Carba NP test the Positive results were observed at 1 to 2 hours. The most interesting aspect of this method is that the colour changed from red to orange for OXA type (positive result). About the modified Hodge test, the presence of a carbapenemase is revealed by deformation of the zone of inhibition due to the enzymatic activity around the antibiotic close to the suspect strain. By contrast, the EDTA test was observed negative which confirmed that the carbapenemase produced by these two strains of *E. Coli* are OXA type which is not inhibited by EDTA.

Resistance-gene determination:

The presence of the carbapenemase blaOXA-48 identified in this study was confirmed by real-time PCR and after was verified and investigated by standard PCR followed by sequencing. In addition, the two isolates of *E. Coli* were revealed producing the blaTEM-1 gene. These two isolates were resistant to beta-lactams, with an ertapenem MIC of 4mg/L and they presented a resistance to aminoglycosides and fluoroquinolones. They were susceptible only to imipenem and colistin.

Strain typing:

According to multilocus sequence typing (MLST) with seven housekeeping genes (*adhA*, *fumC*, *gyrB*, *icd*, *mdh*, *purA* and *recA*), as described at the *E. Coli* MLST Database (<http://mlst.warwick.ac.uk/mlst/dbs/Ecoli>), The results revealed that the two strains of *E. Coli* carbapenemase-positive isolated from urine sample at the university hospital of constantine from two different patients belong to sequence type 38 (ST38). Which was different from the OXA-48 ST5 producing *E. Coli* strain isolated from patient hospitalised at the Military Hospital of Constantine in Algeria [22]. This is the first reported ST38 *E. Coli* strain producing the OXA-48 carbapenemase-encoding gene. There is no report from Algeria that describes the ST38 observed in these strains producing OXA-48.

Transferability of antibiotic resistance:

The transferability of the antibiotic resistance genes was carried out by a conjugation experiment between the donor isolates and azide-resistant *E. Coli* strain J53 as a recipient. The antibiogram of the two isolates of *E. Coli* producing the blaOXA-48 ST38 gene and their transconjugants was determined by disk diffusion method on Mueller-Hinton agar. The inhibition zone diameters were measured and interpreted according to recommendations of the Antibiotic Committee of the French Society for Microbiology CASFM 2015. The extraction of the plasmid and the antibiotic susceptibility results which were negative showed that these resistance genes are chromosomally encoded as previously reported that the OXA carbapenemase genes have been described to be associated with both bacterial chromosomes and plasmids [31].

Discussion:

Carbapenems belong to the most used group of antibiotics (beta - lactams) against the disease infectieuses. The emergence of bacteria resistant to this important group of antibiotics are jeopardizing the use of carbapenems [23] The prevalence of carbapenemase production among Enterobacteriaceae varies from country to country as well as

between different institutions within the country. The carbapenemases OXA-48 are emerging as an important threat. They have first been identified in Turkey [7]. Then, this gene has been identified in many other countries, most often in *K. Pneumoniae* isolates.

OXA-48 was identified in the Middle East and in North African countries, and those countries have been considered as reservoirs of carbapenemase OXA-48 producers [8]. At the same time Poirel et al. Suggested that this carbapenemase may be endemic in Algeria. But its detection has been reported in different surrounding countries (Morocco, Tunisia, and Libya) [11, 24, 25] and in the Mediterranean area (Turkey, France, Spain, Egypt, Italy, and Lebanon) [5, 8, 26]. However, the enzyme was produced by *Klebsiella pneumoniae* strains and not *Escherichia coli*. In 2013 a report from the West of Algeria had revealed that there are no OXA-48 producers in Tlemcen Hospital. However, other types of carbapenemase OXA type have been reported in Algeria, such as the blaOXA-24, blaOXA-23, and blaOXA-58 in Annaba and Tlemcen (Algeria) [12, 27, 28]. Recently, Agabou et al. Reported the presence of the enzyme OXA-48 ST5 in the East of Algeria in one strain of *E. Coli* isolated from surgical infections at the Military Hospital of Constantine, Algeria [22]. So, Today, the novelty in Algerian hospitals is the emergence and dissemination of blaOXA-48 isolated from clinical samples.

In order to gain further understanding of that phenomenon, our report aimed to study the emergence and dissemination and to assess diversity of carbapenemases produced by *Escherichia coli* isolates from patients hospitalised in the university hospital of Constantine (Algeria). So, The here described study investigating a total set of 235 strains of *E. Coli* isolated from patients hospitalised at the University Hospital of Constantine, indicates for the first time in the university hospital of Constantine the presence and the spread of blaOXA-48 on two isolates of *E. Coli* derived from urine samples, and the study of the sequence type had demonstrated that the two isolates carbapenemase OXA-48 producers belong to ST38. In addition, no reports are available on isolates of OXA-48 ST38 producing *E. Coli* from Algeria.

To the best of our knowledge, and according to data in the literature, we report here the first blaOXA-48 ST38 in *E. Coli* from Algeria. The ST38 had been previously detected in the UK, France, Finland, Lebanon and Egypt [29, 30]. This report documents, for the first time, the emergence of the blaOXA-48 ST38 in Algeria.

In conclusion, MLST typing revealed the variety of sts in *E. Coli* producers of blaOXA-48 gene in Algeria. Which confirms the serious problem of the emergence and the genetic diversity of these enzymes and suggests the need to detect and to control their dissemination in this country.

Transparency declarations and Conflict of interest: none to declare

All authors have read and approved the manuscript

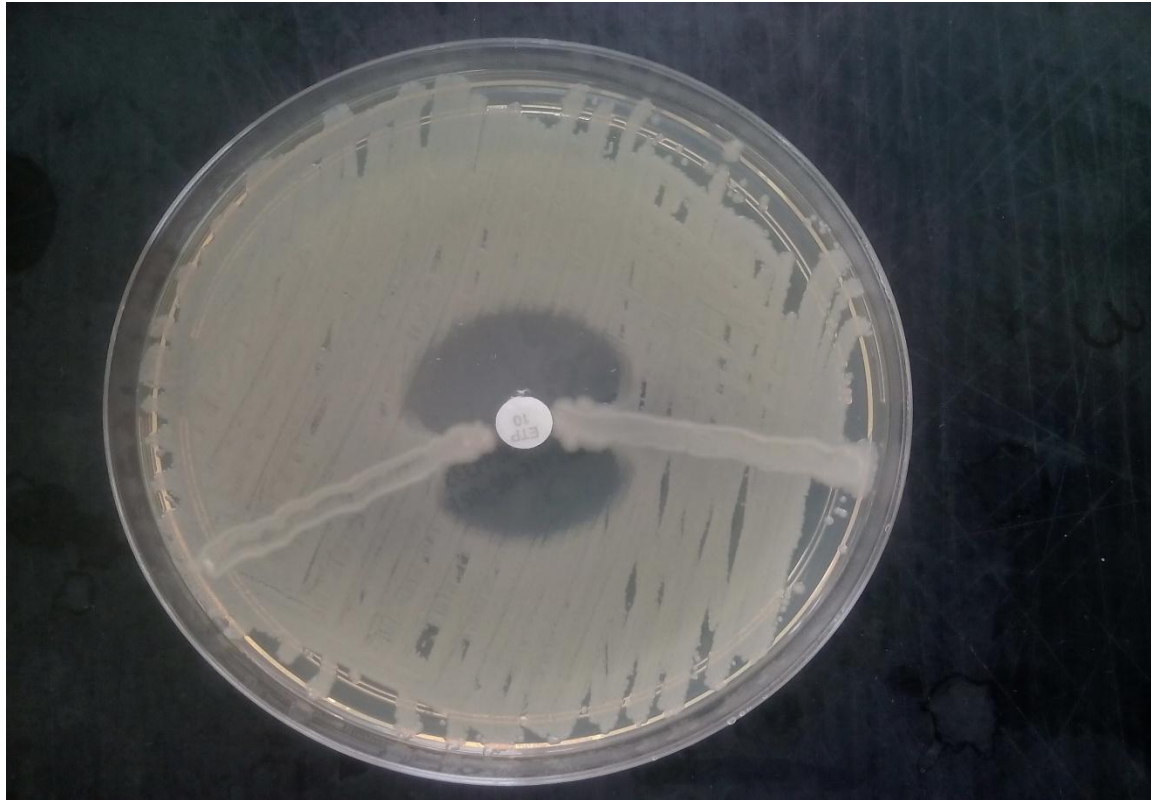


Figure 1: Modified Hodge test positive with strains of E. Coli

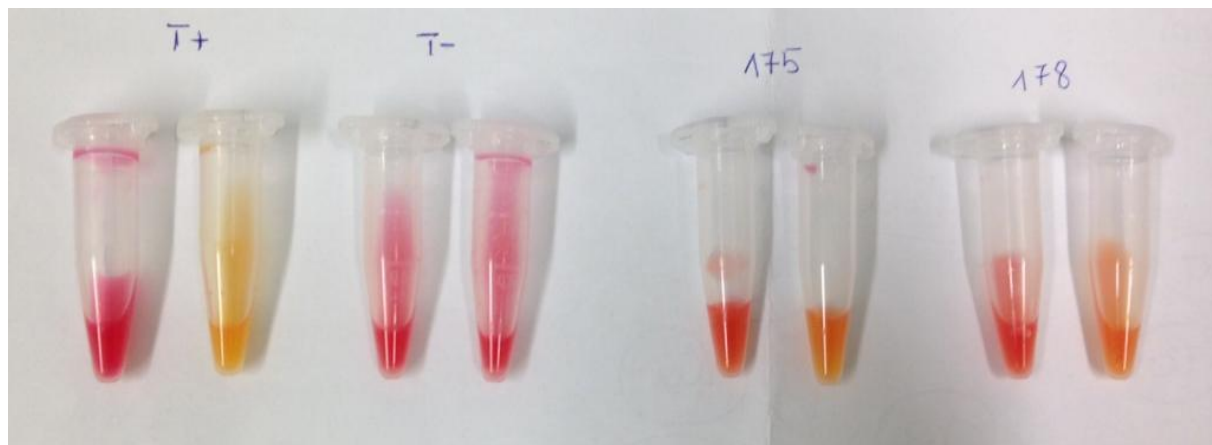


Figure 2 : Carba NP test positive with strains of E. Coli OXA Type

T+ : positive control NDM positive E. Coli

T- : negative control E. Coli J53

178 : E. Coli (EC1) OXA type

175 : E. Coli (EC2) OXA type

Table1. Resistance rate of 235 *Escherichia coli* strains to antibiotics

Antibiotics	Resistant strains of <i>Escherichia coli</i>	
	Number	Percentage %
Amoxicillin	152	64.68
Amoxicillin + clavulanic Ac	140	59.57
Ticarcillin	149	63.40
Piperacillin	91	38.72
Cefazolin	117	49.78
Cefoxitin	12	5.10
Cefotaxim	61	25.95
Aztreonam	50	21.27
Ertapenem	2	0.85
Imipenem	0	0
Gentamycin	27	11.48
Nalidixic acid	80	34.04
Ciprofloxacin	48	20.42
Sulfamethoxazole + Trimethoprim	111	47.23
Colistin	0	0

Table2. Resistance profile of carbapenemase-producing ertapenem-resistant strains

Strains	Ertapenem MIC (mg/l)	Resistance phenotype	Sensitive phenotype
E. Coli (EC1)	4	AX,AMC,TPZ,CFZ,CFPM,FOX,CTX, CAZ, ATM, ERT, AMK, NAL, GN, CIP	IPM, CT
E. Coli (EC2)	4	AX,AMC,TPZ,CFZ,FOX,CTX,CAZ,ATM,ERT, AMK, NAL, GN, CIP	IPM, CT, CFPM

AX : Amoxicillin, AMC : Amoxicillin+clavulanate, TPZ : Piperacillin+tazobactam, CFZ : Cefazolin, CFPM : Cefepim, FOX : Cefoxitin, CTX : Cefotaxim, CAZ : Ceftazidim, ATM : Aztreonam, ERT : Ertapenem, IPM : Imipenem, AMK : Amikacin, NAL : Nalidixic acid, GN : Gentamicin, CIP : Ciprofloxacin, CT : Colistin

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