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Original Article

Characterization of uropathogenic ESBL-producing *Escherichia coli* isolated from hospitalized patients in western Algeria

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Abstract

Introduction: The aim of this study is to assess the prevalence and molecular characterization of uropathogenic Extended spectrum β-lactamases (ESBLs) producing *Escherichia coli*.

Methodology: During 3 years, all hospitalized patients at the University-affiliated hospital of Tlemcen and presenting urinary tract infections caused by *E. coli* were considered as potential study participants. These *E. coli* isolates were examined phenotypically for ESBL production. ESBL strains were subjected to antimicrobial susceptibility testing and were investigated for the presence of plasmid mediated quinolone resistance genes, 16SrRNA methylase genes and virulence genes by using conventional PCR and DNA sequencing. The molecular characterization of ESBL strains was established by phylogenetic grouping method and ERIC-PCR.

Results: The overall prevalence of ESBL was 32.5%. The *bla*$_{\text{CTX-M-15}}$ was the most frequently detected in ESBL isolates, followed by *bla*$_{\text{CTX-M-14}}$, *bla*$_{\text{CTX-M-28}}$, *bla*$_{\text{CTX-M-1}}$ and *bla*$_{\text{SHV-12}}$ respectively. The plasmid-mediated quinolone resistance genes were detected in the 15 ESBL strains with the *aac(6')-Ib-cr* gene was the most detected followed by *qnr*B1 and *qnr*A1 gene respectively. Among the 22 ESBL isolates resistant to gentamicin and amikacin, the 16SrRNA methylase genes were detected in 4 isolates. The *sfa* and *pap* virulent genes were founds in 26% and 22% of isolates receptively. The genotyping analysis of all strains revealed that almost were belonged to phylogenetic groups A_1 and A_0 and fourteen distinct clones.

Conclusion: The emergence of uropathogenic ESBL isolates and the high rate of *bla*$_{\text{CTX-M}}$ are alarming in Algeria. Strict measure must be required to control the further spread of these strains in Algerian hospitals.

Key words: Extended-spectrum β-lactamase; urinary tract infections; *Escherichia coli*.

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Introduction

Extended-spectrum β-lactamases (ESBL) are enzyme identified frequently in *E. coli* and *Klebsiella pneumoniae* and responsible for resistance against penicillins, cephalosporins and aztreonam [1]. The ESBL-producing *E. coli* have widely caused the nosocomial infections which associated with high mortality rates and health care costs [2]. The pathogenicity of these strains is mainly induced by gene-encoding virulence factors, such as adhesion and biofilm formation, production of toxins, and polysaccharide surface coatings.

Urinary tract infections (UTIs) are common diseases in the world, especially for women which occurring in 10% to 20% during their lifetimes [3]. These infections are caused largely by *E. coli* [4]. The phylogenetic analysis of pathogenic ESBL-producing *E. coli* strains revealed that almost all afield to group B2 and, to a lesser extent, group D. Several studies founded that risk factors involved hospital acquired UTIs, include prolonged hospital stay, older age and gender, comorbidity, prior use of antibiotics and frequent use of health resources. ESBL-producing *E. coli* strains are frequently exhibit a multidrug-resistant phenotype, which includes resistance to aminoglycosides and fluoroquinolones [5]. This multidrug resistance making UTIs therapy difficult and promoting greater use of expensive extended spectrum antibiotics.

In correlation with an extensive use of β-lactam antibiotics over the last decades, various β-lactamases types and mutants have emerged in hospital and community settings. Initially, the first ESBLs identified

due to mutations in the genes encoding the common plasmid-mediated SHV-1, TEM-1, or TEM-2 β-lactamases [6]. These mutations are susceptible to hydrolyze extended-spectrum antibiotics. More than 180 TEM-type and 130 SHV-type β-lactamases had been identified worldwide (lahey.org/Studies/). Actually, CTX-M enzymes that preferentially hydrolyze and confer resistance to cefotaxime are being found in several continents around the world. More than 100 CTX-M, divided into five groups (CTX-M-1, 2, 8, 9 and 25) had been identified (lahey.org/Studies/).

In Algeria, the isolates *E. coli* and *K. pneumoniae* producing ESBL were the most predominant among multi-drug resistant clinical isolates. The β-lactamase genes detected in Algerian hospitals isolates were $bla_{\text{CTX-M}}$, bla_{TEM}, bla_{SHV}, bla_{DHA}, bla_{PER} , bla_{VEB} and bla_{OXA} types. Furthermore, the Algerian ESBL producer isolates, especially, *Enterobacteriaceae* producer of CTX-M enzyme were manly harboured of plasmid mediated-quinolones resistance determinants.

Actually, it was observed an increase in the failure of treatment of both complicated and uncomplicated UTIs, which continuously increasing morbidity and mortality among patients. In this study, we investigated the prevalence of uropathogenic ESBL-producing *E. coli* isolated from hospitalized patients at University-affiliated hospital of Tlemcen; their antimicrobial-resistance profiles, molecular typing of ESBLs enzymes and the genetic diversity among clinical ESBL-producing *E. coli* strains isolated. Also, this study aims to analyses various risk factors associated with the occurrence of infections by ESBL-producing strains.

Methodology

Setting

This study was conducted at the University-affiliated hospital of Tlemcen (public institution) located in Tlemcen city (situated at 600 km west of the capital Algiers), with 800 bed teaching hospital distributed in 25 medical and surgical units, including two adult intensive care units (ICUs) with a total of 14 beds. The university-affiliated Hospital of Tlemcen, receives approximately 136,000 patients for consultations and 6000 admissions per year.

Data collection and bacterial strains

All the hospitalized patients presenting UTI and infected by *E. coli* from January 2011 to December 2013 were included. The medical records of these patients were retrieved and reviewed. Information was obtained about basic demographic characteristics (age, sex and pre-infection hospital stay) as well as co-morbid diseases (surgical intervention, renal diseases, respiratory diseases, central nervous diseases, and others), use of urinary catheters, intensive care unit admission, previous hospitalization, and length of hospital stay. The diagnosis of nosocomial infection was established according to the Center for Disease Control (CDC) criteria.

Previous hospitalization was defined as hospitalization at University-affiliated hospital of Tlemcen within 30 days prior to the current admission. Recent surgery was defined as any surgical procedure performed in the operating room within 30 days of entry in the study. The origin of isolate was accepted as nosocomial if the strain was isolated more than one week after hospitalization.

E. coli isolated from urine were identified using API 20E system (Biomerieux, Marcy l'Etoile, France). Prior to their testing, all the isolates were stored in 15% glycerol-supplemented Luria-Bertani medium (Bio-Rad, Marnes-la-Coquette, France) at -80°C.

Antimicrobial drug susceptibility testing

Antimicrobial drug susceptibility was determined by the disk-diffusion method on Mueller-Hinton (MH) agar plates (HiMedia, Mumbai, India), as recommended by the CLSI [7]. The following antimicrobial agents (Bio-Rad, Marnes-la-Coquette, France) were tested: amoxicillin (25 μg), amoxicillin/ clavulanic acid (30 μg), ticarcillin (75 μg), ticarcilline/ clavulanic acid (85 μg), piperacillin (75 μg), piperacillin/ tazobactam (85 μg), cephalotin (30 μg), cefotaxime (30 μg), ceftazidime (30 μg), cefepime (30 μg), imipenem (10 μg), aztreonam (30 μg), cefoxitin (30 μg), gentamicin (15 μg), amikacin (30 μg), nalidixic acid (30 μg), and ciprofloxacin (5 μg).

Multidrug-resistants (MDR) were defined as those resistant to at least two of the antibiotic classes in addition to the β-lactams. *E. coli* ATCC 25922 (American Type Culture Collection, Manassas, USA) was used as quality control strain.

Phenotypic detection of ESBL production

According to CLSI guidelines [7], the ESBL production was detected by the double-disc synergy test using cefotaxime (30 μg) disks, with and without clavulanic acid (10 μg). The *E. coli* test strains were inoculated into Mueller-Hinton agar at a 0.5 McFarland standard, followed by incubation at 35°C for 16 to 18 hours. Diameters of inhibition zones were measured with a standard caliper (Flower Company Inc., Newton, MA, USA). A difference in inhibition zones of ≥5 mm for cefotaxime-clavulanic acid combination versus the

corresponding cefotaxime alone was considered indicative of ESBL production.

The standard strains *E. coli* ATCC 25922 and *K. pneumoniae* ATCC 700603 (American Type Culture Collection, Manassas, USA) were used as negative and positive controls of ESBL production, respectively.

Preparation of DNA template for PCR

DNA templates for Polymerase Chain Reaction (PCR) process were generated by suspending 5 colonies of an overnight culture of *E. coli* isolates growing on Luria Bertani agar (Bio-Rad, Marnes-la-Coquette, France) in 500µL of DNase- and RNase-free water (Invitrogen, Paisley, UK). The suspension was boiled at 100°C for 10 minutes in thermal block (Bioblock Scientific, Illkirch, France), then centrifuged at 19000g for 5 minutes. An aliquot of 1 µL of the supernatant was used as DNA template for PCR

Detection of β-lactamases encoding genes

All ESBL-producing *E. coli* isolates were screened by PCR for the following β-lactamase-encoding genes: $bla_{\text{CTX-M}}$ phylogenetic lineage groups 1, 2, 8, 9 and 25, bla_{TEM}, bla_{SHV}, bla_{OXA}, bla_{PER} and bla_{VEB} genes as described by Barguigua *et al*. [8]. ESBL-producing *E. coli* resistance to cefoxitin antibiotic were further tested by using primers specific for plasmid-mediated AmpC β-lactamases encoding genes as described by Pérez-Pérez and Hanson [9].

Detection of plasmid mediated quinolone resistance genes

All ESBL-producing strains were screened by multiplex PCR for *qepA* and *qnr* genes (*qnrA*, *qnrB, qnrD, qnrC* and *qnrS*) as previously reported [8]. PCR amplification of *aac(6')-Ib-cr*, a fluroquinolone-modifying aminoglycosid acetyltransferase, was performed using primers that amplify all *aac(6')-Ib* variants. Isolates positive for *aac(6')-Ib* were sequenced to identify *aac(6')-Ib-cr* as described by Barguigua *et al*. [8].

Detection of 16S rRNA methylase genes

To detect 16S rRNA methylase gene alleles (*armA*, *rmtA*, *rmtB* and *rmtC*), all ESBL producers isolates that were resistant to gentamicin or amikacin were included for study. The PCR primers and method used were as previously reported [10].

Detection of virulence genes

Multiplex PCR was used for the amplification of genes encoding pyelonephritis associated pili (*pap* genes), S-family adhesions (*sfa* gene), hemolysin (*hly* gene), and cytotoxic necrotizing factor 1 (*cnf1*) as previously described [11].

Phylogenetic genotyping

Phylogenetic grouping of the *E. coli* isolates was determined by a PCR-based method developed by Clermont et *al*. [12] that uses a combination of 3 DNA markers (*chuA*, *yjaA*, and an anonymous DNA fragment, TspE4.C2). Strains were assigned to phylogenetic groups on the basis of presence or absence of the 3 DNA fragments: *chuA*-, TspE4.C2-, group A; *chuA*-, *yjaA*-, Tspe4.C2+, group B1; *chuA*+, *yjaA*+, group B2; *chuA*+, *yjaA*-, group D.

To increase the strains discrimination, subgroups or phylotypes were determined as follows: group A subgroup A_0, *chuA*-, *yjaA*-, Tspe4.C2-; group A subgroup A_1, *chuA*-, *yjaA*+, Tspe4.C2-; group B2 subgroup $B2_2$, *chuA*+, *yjaA*+, Tspe4.C2-; group B2 subgroup $B2_3$, *chuA*+, *yjaA*+, Tspe4.C2+; group D subgroup D_1, *chuA*+, *yjaA*-, Tspe4.C2- and group D subgroup D_2,*chuA*+, *yjaA*-, Tspe4.C2+ [13].

All primers used in this work are shown in Table 1.

Sequencing of resistance genes

All amplified products obtained were sequenced to validate their identities. Both strands of the purified amplicons were sequenced with a Genetic Analyzer 3130×1 sequencer (Applied Biosystems, Foster City, CA, USA), with the same primers used for PCR amplification. The nucleotide and deduced protein sequences were analysed with software available over the Internet at the National Center for Biotechnology Information website (www.ncbi.nlm.nih.gov).

Enterobacterial repetitive Intergenic Consensus (ERIC)

ERIC analysis was performed using the primer ERIC-2 as previously reported [14]. Each PCR reaction was carried out in a 25 µL volume using 1.5 U of *Taq* DNA polymerase (Promega, Madison, Wis, USA) in the reaction buffer provided by the manufacturer containing 2.5 mM of $MgCl_2$, 50 µM of each deoxynucleoside triphosphate, 0.3 µM of the selected primer and 5 µL of the DNA template. Aliquots (10 µL) of each PCR product were subjected to an electrophoresis on 1.5% agarose gel.

Statistical analyses

The relation between the ESBL-producing *E. coli* strains and possible risk factors was evaluated according to the epidemiological data collected.

Table1. Primers used for PCR amplification.

Gene	Primer	Primer sequence (5'→3')*	Reference
qnrA	qnrA(+)	TTCTCACGCCAGGATTTGAG	[8]
	qnrA(-)	TGCCAGGCACAGATCTTGAC	
qnrB	qnrB(+)	TGGCGAAAAAAATT(GA)ACAGAA	[8]
	qnrB(-)	GAGCAACGA(TC)GCCTGGTAG	
qnrS	qnrS (+)	GACGTGCTAACTTGCGTGAT	[8]
	qnrS (-)	AACACCTCGACTTAAGTCTGA	
qnrC	qnrC(+)	GGGTTGTACATTTATTGAATC	[49]
	qnrC(-)	TCCACTTTACGAGGTTCT	
qnrD	qnrD(+)	CGAGATCAATTTACGGGGAATA	[49]
	qnrD(-)	AACAAGCTGAAGCGCCTG	
qepA	qepA(+)	TGGTCACGCCATGGACCTCA	[50]
	qep(-)	TGAATTCGGACACCGTCTCCG	
aac(6')-Ib	aac(6')-Ib(+)	ATGACTGAGCATGACCTTG	[8]
	aac(6')-Ib(-)	AACCATGTACACGGCTGG	
***bla*CTX-M group1**	CTX-M1(+)	GGTTAAAAAATCACTGCGTC	[8]
	CTX-M1(-)	TTGGTGACGATTTTAGCCGC	
***bla*CTX-M group2**	CTX-M2(+)	ATGATGACTCAGAGCATTCG	[8]
	CTX-M2(-)	TGGGTTACGATTTTCGCCGC	
***bla*CTX-M group9**	CTX-M9(+)	ATGGTGACAAAGAGAGTGCA	[8]
	CTX-M9(-)	CCCTTCGGCGATGATTCTC	
***bla*CTX-M-8**	CTX-M-8(+)	TCGCGTTAAGCGGATGATGC	[8]
	CTX-M-8(-)	AACCCACGATGTGGGTAGC	
***bla*CTX-M-25**	CTX-M-25(+)	GCACGATGACATTCGGG	[8]
	CTX-M-25(-)	AACCCACGATGTGGGTAGC	
***bla*TEM**	a-216	ATAAAATTCTTGAAGACGAAA	[8]
	a-217	GACAGTTACCAATGCTTAATCA	
***bla*SHV**	Os-5	CGCCGGGTTATTCTTATTTGTCGC	[8]
	Os-6	CGCCGGGTTATTCTTATTTGTCGC	
***bla*PER**	per (+)	CCTGACGATCTGGAACCTTT	[8]
	per(-)	GCAACCTGCGCAAT(GA)ATAGC	
***bla*VEB**	veb (+)	ATTTCCCGATGCAAAGCGT	[8]
	veb(-)	TTATTCCGGAAGTCCCTGT	
***bla*GES**	GES(+)	ATGCGCTTCATTCACGCAC	[8]
	GES(-)	CTATTTGTCCGTGCTCAGGA	
***bla*FOX**	foxM(+)	AACATGGGGTATCAGGGAGATG	[9]
	foxM(-)	CAAAGCGCGTAACCGGATTGG	
***bla*ACC**	accM(+)	AACAGCCTCAGCAGCCGGTTA	[9]
	accM(-)	TTCGCCGCAATCATCCCTAGC	
***bla*EBC**	ebcM(+)	TCGGTAAAGCCGATGTTGCGG	[9]
	ebcM(-)	CTTCCACTGCGGCTGCCAGTT	
***bla*MOX**	moxM(+)	GCTGCTCAAGGAGCACAGGAT	[9]
	moxM(-)	CACATTGACATAGGTGTGGTGC	
***bla*CIT**	citM(+)	TGGCCAGAACTGACAGGCAAA	[9]
	citM(-)	TTTCTCCTGAACGTGGCTGGC	
***bla*DHA**	dhaM(+)	AACTTTCACAGGTGTGCTGGGT	[9]
	dhaM(-)	CCGTACGCATACTGGCTTTGC	
armA	armA(+)	TATGGGGGTCTTACTATTCTGCCTAT	[10]
	armA(-)	TCTTCCATTCCCTTCTCCTTT	
rmtA	rmtA(+)	CTAGCGTCCATCCTTTCCTC	[10]
	rmtA(-)	TTTGCTTCCATGCCCTTGCC	
rmtB	rmtB(+)	TCAACGATGCCCTCACCTC	[10]
	rmtB(-)	GCAGGGCAAAGGTAAAATCC	

Table1 (continued). Primers used for PCR amplification.

Gene	Primer	Primer sequence (5'→3')*	Reference
rmtC	rmtC(+)	GCCAAAGTACTCACAAGTGG	[10]
	rmtC(-)	CTCAGATCTGACCCAACAAG	
chuA	chuA .1	GACGAACCAACGGTCAGGAT	[12]
	chuA. 2	TGCCGCCAGTACCAAAGACA	
yjaA	yjaA.1	TGAAGTGTCAGGAGACGCTG	[12]
	yjaA.2	ATGGAGAATGCGTTCCTCAAC	
TspE4.C2	TspE4.C2.1	GAGTAATGTCGGGGCATTCA	[12]
	TspE4.C2.2	CGCGCCAACAAAGTATTACG	
hly	hly(+)	AACAAGGATAAGCACTGTTCTGGCT	[11]
	hly(-)	ACCATATAAGCGGTCATTCCCGTCA	
cnf-1	cnf-1(+)	AAGATGGAGTTTCCTATGCAGGAG	[11]
	cnf-1(-)	CATTCAGAGTCCTGCCCTCATTAT	
pap	pap(+)	GACGGCTGTACTGCAGGGTGTGGCG	[11]
	pap(-)	ATATCCTTTCTGCAGGGATGCAATA	
sfa	sfa(+)	CTCCGGAGAACTGGGTGCATCTTAC	[11]
	sfa(-)	CATCAAGCTGTTTGTTCGTCCGCCG	
ERIC	ERIC-2	AAGTAAGTGACTGGGGTGAGC	[14]

Data were entered into a database using the SPSS 20.0 for Windows (SPSS Inc, Chicago, USA), the χ^2 test and the independent sample *t* test was used for categorical and continuous variables, respectively. A stepwise multivariate logistic regression was conducted to examine the association of risk factors controlling for potential confounders. The logistic model included all variables for which a *p* value of < 0.1 was obtained in the multivariate analysis. A *p* value of < 0.05 was considered as significant.

Results

A total of 441 patients having symptomatic UTIs were included in this study. Eighty-three patients presented with *E. coli* infection (18.82%) with 27 (32.5%) patients with ESBL-producing *E. coli*

Table 2. Clinical background characteristics of patients infected and not infected with extended-spectrum β-lactamase-producing *E. coli.*

characteristic	ESBL (n = 27)	Non-ESBL (n = 56)	*p*-value	OR	95% CI
Age-years					
Mean	50.58 ± 21.37	43.48 ± 18.39	0.16		-2.78-15.59
Median	40	36			
Gender					
Female	17 (62.96%)	39 (69.64%)	0.54	0.74	0.28-1.94
Male	10	17			
Sex-ratio	0.58	0.43	0.80		-3.15-2.45
Duration of hospitalization (days)	9.10 ± 6.11	10.14 ±5.94			
Previous urinary infection	12 (44.44%)	23(41.07%)	0.77	1.14	0.45-2.90
Hospital admission ward					
Urology	10 (37.07%)	23 (41.07%)	0.72	0.84	0.32-2.17
Maternity	9 (33.33%)	16 (28.57%)	0.65	1.25	0.46-3.35
Medical	8 (29.62%)	17 (30.35%)	0.94	0.96	0.35-2.63
Type of invasive device					
Central venous catheter	5 (18.51%)	25(44.64%)	0.02	0.28	0.09-0.85
Previous hospitalization	15 (55.55%)	23(41.07%)	0.21	1.80	0.70-4.53
Coexisting diseases					
Diabetes	8(29.62%)	14(25%)	0.51	0.68	0.21-2.14
Infected wound	0	5 (8.92%)	0.17	----	---
hypertension	4(14.81%)	7 (12.5%)	0.41	1.77	0.43-7.22
urethral stricture	3(11.11%)	1 (1.78%)	0.63	6.87	0.68-69.50
anaemia	2 (7.40%)	1 (1.78%)	0.19	4.40	0.38-50.81
Renal disease	3 (11.11%)	7(12.5%)	0.85	0.87	0.20-3.68
Cancer	5 (18.51%)	12 (21.42%)	0.90	0.92	0.28-3
Other	2 (7.40%)	9 (16.07%)	0.44	2.16	0.28-16.12

infection. During the study period (2011-2013), we note that the highest frequency of ESBL producing *E. coli* isolation was in 2013 (37.84%) (Figure 1).

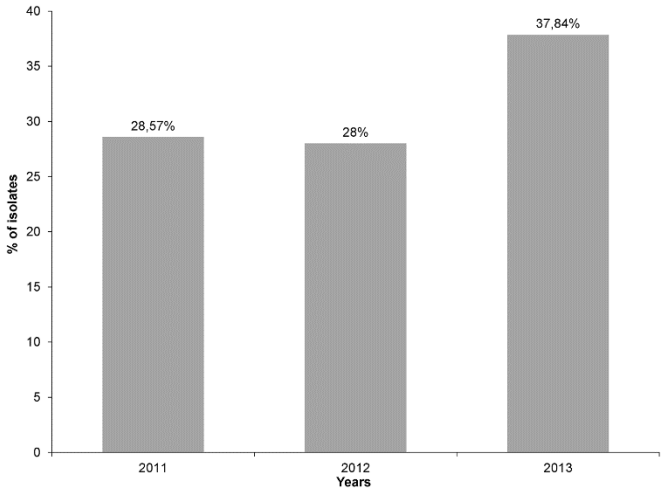
The median age was 40 in the ESBL group and 36 in the non-ESBL group, with no significant difference, using parametric (for mean) or non-parametric (for median) tests. The duration of hospitalization was slightly different between the groups (ESBL and non-ESBL), although it had a tendency to be prolonged in the non-ESBL group (Table 2).

The most common coexisting disease in two groups (ESBL and non-ESBL) included the diabetes, cancer and hypertension. Among the ESBL group, the frequency of previous hospitalization (55.5%) was higher than non-ESBL group (41.07%). Even though several variables were compared (ESBL vs. non-ESBL), central venous catheter use was statistically significant (Table 2). In the multivariate analysis, none of the risk factors for ESBL were identified.

The antimicrobial susceptibility testing was done by disc diffusion method to 83 clinical isolates of *E. coli*. The resistant rates for β-lactam antibiotics including amoxicillin, ticarcillin,

cefalotin, piperacillin and amoxicillin/clavulanic acid were more than 64% in all tested clinical strains. The resistant rates for non-β-lactam antibiotics including nalidixic acid, gentamicin, ciprofloxacin, and amikacin were 38.55%, 22.89%, 21.68% and 18.07%, respectively.

Figure 1. Evolution of frequency of ESBL-producing *E. coli* isolated from UTI during three years.



The degrees of antibiotic resistance for all antibiotics tested were significantly higher in the ESBL isolates, compared with non-ESBL isolates (Table 3). Imipenem was the most effective antibiotic against ESBL isolates with a susceptibility of 100%. Non-ESBL isolates were significantly more resistant to piperacillin/ tazobactam (33.29% vs. 0%), amoxicillin/ clavulanic acid (75% vs. 44.44%), ticarcillin/clavulanic acid (44.64% vs. 29.62%) compared to ESBL isolates.

Table 3. Resistance rate of *E. coli* strains to different antimicrobial agents.

Antibiotics	Proportion of resistance (%), (No. of resistant strains)			p-value
	All strains % (n = 83)	ESBL producers % (n = 27)	Non-ESBL producers % (n = 56)	
AML	85.54 (71)	100 (27)	78.57 (44)	< 0.01
TIC	77.10 (64)	100 (27)	66.07 (37)	< 0.001
KF	72.2 (60)	100 (27)	58.92 (33)	< 0.00001
PRL	75.90 (63)	100 (27)	64.28 (36)	< 0.001
TZP	22.89 (19)	0	33.92 (19)	< 0.00001
AMC	65.06 (54)	44.44 (12)	75 (42)	< 0.01
TCC	39.97 (33)	29.62 (8)	44.64 (25)	< 0.001
CTX	57.83 (48)	100 (27)	37.5 (21)	< 0.00001
CAZ	57.83 (48)	100 (27)	37.5 (21)	< 0.00001
ATM	56.62 (47)	100 (27)	35.71 (20)	< 0.00001
FEP	39.75 (33)	100 (27)	10.71 (6)	< 0.00001
FOX	31.32 (26)	22.22 (6)	35.71 (20)	NS
IMP	0	0	0	
AK	18.07 (15)	48.14 (13)	3.57 (2)	< 0.00001
CN	22.89 (19)	40.74 (11)	14.28 (8)	< 0.01
NA	38.55 (32)	55.33 (15)	30.35 (17)	< 0.05
CIP	21.68 (18)	29.62 (8)	17.85 (10)	NS

AML: Amoxicillin, AMC: amoxicillin/ clavulanic acid, TIC: ticarcillin, TCC: ticarcillin, ticarcilline/ clavulanic acid, PRL: piperacillin, TZP: piperacillin/ tazobactam, KF: cephalotin, CTX: cefotaxime, CAZ: ceftazidime, FEP: cefepime, IMP: imipenem, ATM: aztreonam, FOX: cefoxitin, CN: gentamicin, NA: nalidixic acid, AK: amikacin CIP: ciprofloxacin.

Table 4. Characteristics of the ESBL-producing *E. coli* strains studied.

Code	Date of isolation	Ward	Sex/age	ESBL gene	Others β-lactamase genes	PMQR	16S rRNA methylase gene	virulence genes	Phylogenetic group	ERIC pattern
ec01	01/04/2013	Maternity	F/35	*bla*$_{CTX-M-15}$	*bla*$_{TEM-1}$	*aac(6')-Ib-cr*			A_1	A
ec03	01/04/2013	Maternity	F/31	*bla*$_{CTX-M-15}$, *bla*$_{CTX-M-14}$	*bla*$_{TEM-1}$				B1	B
ec06	01/05/2013	Maternity	F/30	*bla*$_{CTX-M-15}$	*bla*$_{TEM-1}$		*armA*		A_0	C
ec04	01/03/2013	Maternity	F/37	*bla*$_{CTX-M-15}$	*bla*$_{TEM-1}$	*qnrB1, aac(6')-Ib-cr*		*pap*	A_1	C
ec05	01/04/2013	Maternity	F/38	*bla*$_{CTX-M-15}$, *bla*$_{CTX-M-14}$	*bla*$_{TEM-1}$	*aac(6')-Ib-cr*		*sfa*	A_1	C
ec18	01/02/2012	Internal medicine	F/65	*bla*$_{CTX-M-15}$	*bla*$_{TEM-1}$	*qnrB1*			A_1	C
ec24	01/06/2013	maternity	F/39	*bla*$_{CTX-M-15}$	*bla*$_{TEM-1}$				A_0	E
ec08	11/11/2011	Maternity	F/31	*bla*$_{CTX-M\ 15}$	*bla*$_{TEM-1}$	*aac(6')-Ib-cr*	*armA ,rmtB*	*sfa*	A_1	E
ec28	01/02/2011	Urology	F/62	*bla*$_{CTX-M-15}$	*bla*$_{TEM-1}$	*qnrB1, aac(6')-Ib-cr*			D_1	E
ec12	01/11/2012	Urology	M/63	*bla*$_{CTX-M-15}$	*bla*$_{TEM-1}$				A_0	F
ec09	01/02/2011	Urology	M/91	*bla*$_{CTX-M-15}$	*bla*$_{TEM-1}$	*aac(6')-Ib-cr*	*armA, rmtB*	*sfa*	A_1	F
ec10	01/06/2013	Urology	M/57	*bla*$_{CTX-M-15}$	*bla*$_{TEM-1}$			*pap*	B1	F
ec11	11/11/2011	Urology	M/69	*bla*$_{CTX-M-15}$	*bla*$_{TEM-1}$	*aac(6')-Ib-cr*			$B2_3$	F
ec14	01/01/2012	Urology	M/90	*bla*$_{CTX-M-15}$	*bla*$_{TEM-1}$	*aac(6')-Ib-cr*		*sfa,*	A_1	G
ec16	01/06/2012	Internal medicin	F/95	*bla*$_{CTX-M-15}$	*bla*$_{TEM-1}$	*aac(6')-Ib-cr*			B1	H
ec19	01/11/2011	Internal medicin	F/40	*bla*$_{CTX-M-15}$	*bla*$_{TEM-1}$, *bla*$_{MOX}$			*pap*	B1	H
ec20	01/02/2013	Internal medicin	F/37	*bla*$_{CTX-M-1}$	*bla*$_{TEM-1}$			*pap*	$B2_2$	H
ec15	01/06/2013	Urology	M/42	*bla*$_{CTX-M-15}$	*bla*$_{TEM-1}$	*aac(6')-Ib-cr*		*sfa*	$B2_3$	H
ec17	01/12/2012	Internal medicin	F/56	*bla*$_{CTX-M-15}$			*armA*	*pap*	$B2_3$	H
ec25	01/03/2013	Internal medicin	F/55	*bla*$_{CTX-M-15}$	*bla*$_{TEM-1}$				B1	I
ec21	01/05/2013	Internal medicin	F/83	*bla*$_{CTX-M-28}$	*bla*$_{TEM-1}$			*sfa,cf1*	$B2_3$	I
ec22	01/03/2013	Internal medicin	M/35	*bla*$_{CTX-M-15}$	*bla*$_{TEM-1}$				A_0	J
ec23	01/03/2013	Urology	M/30	*bla*$_{CTX-M-15}$	*bla*$_{TEM-1}$	*aac(6')-Ib-cr*			A_1	K
ec29	01/06/2012	Urology	M/79	*bla*$_{CTX-M-15}$, *bla*$_{SHV-12}$	*bla*$_{TEM-1}$	*aac(6')-Ib-cr*		*sfa, pap*	$B2_3$	N
ec27	01/04/2013	Maternity	F/37	*bla*$_{CTX-M-15}$	*bla*$_{TEM-1}$	*qnrB1*			A_0	O
ec26	01/11/2012	Urology	M/58	*bla*$_{CTX-M-15}$	*bla*$_{TEM-1}$, *bla*$_{CMY-2}$				A_0	P
ec02	01/02/2013	Maternity	F/22	*bla*$_{CTX-M-15}$	*bla*$_{TEM-1}$	*qnrA1, aac(6')-Ib-cr*			A_1	Q

PCR analysis was performed for all the ESBL isolates. The results of PCR and sequence analysis are summarized in Table 4. The $bla_{\text{CTX-M-15}}$ (n = 25) was the most frequently detected in ESBL isolates, followed by $bla_{\text{CTX-M-14}}$ (n = 2), $bla_{\text{CTX-M-28}}$ (n = 1), $bla_{\text{CTX-M-1}}$ (n = 1) and $bla_{\text{SHV-12}}$ (n = 1) respectively. Three ESBL isolates were identified harbouring two ESBL genes with $bla_{\text{CTX-M-15}}$ + $bla_{\text{CTX-M-14}}$ (n = 2), $bla_{\text{CTX-M-15}}$ + $bla_{\text{SHV-12}}$ (n = 1) combination. None of the ESBL isolates gave positive PCR results for ESBL encoding genes; bla_{PER}, bla_{VEB} and $bla_{\text{CTX-M}}$ phylogenetic lineage groups 2, 8 and 25. The $bla_{\text{TEM-1}}$ was detected in 26 (96%) of ESBL strains.

The cephalosporinases CMY-2 and MOX types were found in two ESBL isolates. These genes has been presented with the following combinations: $bla_{\text{CMY-2}}$ + $bla_{\text{CTX-M-15}}$ + $bla_{\text{TEM-1}}$ and bla_{MOX} + $bla_{\text{CTX-M-15}}$+ $bla_{\text{TEM-1}}$.

The PMQR genes were found in the 15 ESBL strains examined with the *aac(6')-Ib-cr* gene (n = 13) was the most detected followed by *qnr*B1 (n = 4) and *qnr*A1 (n = 1) gene respectively (Table 4). Three isolates of ESBL producers were co-harboured two PMQR genes with the following combination: *qnr*B1+ *aac(6')-Ib-cr* (n = 2) and *qnr*A1+*aac(6')-Ib-cr* (n = 1).

Among the 22 ESBL isolates resistant to gentamicin and amikacin, the 16S rRNA methylase genes were detected in 4 isolates (18.18%). The *armA*+ *rmtB* combination was found in two isolate and *armA* alone in others two strains (Table 4).

The research of virulence factors such as *pap, sfa*, *hly*, and *cnf1* by the multiplex PCR showed that the prevalence of virulent genes ranged from 4% to 26%: 4% for *cnf1,* 22% for *pap* and 26% for *sfa*. The phylogenetic analysis of all ESBL strain revealed that almost of the strains studied were belonged to groups A1 (n = 9) and A0 (n = 6), followed by B1 (n = 5), B2 (n = 6) and D1 (n = 1).

In the genotyping analysis, the ERIC-PCR patterns of the ESBL-producing isolates revealed fourteen distinct clones (Table 4). Nine of these clones (clones A, B, G, J, K, N, O, P and Q) were represented by a single isolate. Clone F was represented by four strains (ec12, ec9, ec10 and ec11) isolated from old male patients hospitalized in urology ward. Clones C (4 strains) and clone E (3 strains) were mostly represented by strains identified in maternity ward. The patients hospitalized in the internal medicine were frequently infected by strains included in clone H (4 strains) and clone I (2 strains).

Discussion

At university-affiliated hospital of Tlemcen, the rate of UTIs is much noticed in the maternity and urology wards. The recognized risk factors of UTIs in the literature were feminine sex, age over 65 years, and a recent hospitalization under six months [15,16]. In contrast other studies showed that there are no significant difference between men and women [15,16]. However these results are concordant with those obtained in this study, which we found that diabetes is no longer considered a risk complicating factor $p \leq 0.001$ [15,16]. Other causes may play an important role in UTIs such the non-compliance to hygiene rules, notably washing and hand disinfection by health care personnel, which is an important risk factor for the transmission of nosocomial infections.

In recent years, the increase of antibiotic resistance has threatened the entire world. The production of β-lactamase, which hydrolyses and inactivate β-lactam antibiotics, become one of the most important resistance mechanisms of several bacterial species, mainly in *Enterobacteriaceae* family. Bacterial resistance to extended spectrum β-lactam antibiotics is increasingly associated with ESBL [17].

The overall prevalence of ESBLs production varied considerably according to geographic areas and hospital structures. ESBLs prevalence rate in this study is still strong compared to that reported in northern Algeria (19.9%) [18] and Tunisia (20.2%) [19]. However, higher prevalence rates of ESBLs produced by *K. pneumoniae* were detected in South America (45.4% to 51.9%) [20] and Saudi Arabia (55%) [21]. The current study showed that *E. coli* was the commonest cause of UTIs (62%) followed by *K. pneumoniae*. The prevalence of ESBL-producing *E. coli* stains was 32%. This high rate of ESBL producing *E. coli* at university-affiliated hospital of Tlemcen could be attributed to the indiscriminate and widespread use of antibiotics, particularly β-lactam antibiotics which are sold over the counter in pharmacy shops without doctors' prescription.

In this study, antibiotics susceptibility tests showed that ESBL producing *E. coli* isolates were susceptible to imipenem and were significantly multi resistant to all others antibiotic tested (Table 2). Rare use of imipenem in the hospital of Tlemcen could explain the high susceptibility to this antibiotic.

The sensitivity of *Enterobacteriaceae* to ß-lactam antibiotics showed high levels of acquired resistance in these bacteria, especially in hospital setting, mainly due to the extensive use of β-lactam antibiotics, which cause a selection pressure of resistant strains. In addition,

these resistance are also due to plasmids transfer between pathogens [22]. In contrast, Aminoglycosides and Fluoroquinolones antibiotics have an excellent activity against ESBLs producers *Enterobacteriaceae* [23].

The vast majority of *E. coli* strains isolated in our study are sensitive to imipenem, however amoxicillin and cephalothin are frequently resistance because it is the most prescribed in the local market.

The most strains of ESBL-producing *E coli* were resistant to gentamicin, and ciprofloxacin; it supports the work of Tolun *et al*. [24] and Alhambra et *al*. [25]. The mechanisms of this resistance are not yet clearly established, but some authors suggest the co-transmission ESBL and other antibiotic resistance genes by the same conjugative plasmid [22].

In this study, amongst the 27 CTX-M-producing *E. coli* isolates, CTX-M-15 was the major ESBL (25 isolates) followed by CTX-M-14 (2 isolates). CTX-M-14 and CTX-M-15 enzymes were commonly detected in Algerian hospitals [26,27]. Many studies have reported that CTX-M-15 type enzymes were the predominant type of ESBL in hospital-acquired UTIs [28,29]. Most of the ESBL-*Enterobacteriaceae* isolates were resistant to the other classes of antibiotics, in particular fluoroquinolones, which are commonly prescribed by general practitioners against UTIs. However, increased fluoroquinolones resistance has now been associated with ESBL isolates [29]. This increase has apparently occurred in parallel with the emergence of plasmid-mediated fluoroquinolone resistance mechanisms [30]. Furthermore, our study has revealed the presence of plasmid-mediated broad-spectrum resistances co-existing with ESBLs, such as *qnrB*, *qnrS* and *aac(6')-Ib-cr* which confers quinolone resistance.

Several studies have shown a close association between the CTX-M and *qnr* determinants. Iadadene *and al.* [27] revealed in their study, the co-producing of *qnrB1* and *qnrS1* with CTXM- 15 and SHV-12 in Algerian isolates of *E. cloacae*. The presence of *qnr* and *aac (6')-Ib-cr* genes in clinical isolates of *E. coli* and *K. pneumoniae* has been reported worldwide [22,30]. A large number of studies have also shown the presence of *qnr* genes along with resistance to various β-lactamase, including the AmpC and ESBL [27,31]. In Algeria, it was detected for the first time in 2009 in a strain of *E. cloacae* [32] and among *Enterobacteriaceae* strains in the community [33]. The prevalence of *aac(6')-Ib-cr* in our study is in agreement with previous studies reporting high rates of the *aac(6')* allele and its association with ESBL, particularly CTX-M [34,35]. The majority of *E. coli* strains positive for *aac(6')-Ib-cr* were often co-expressed an ESBL CTXM-1 (75 %). There is one other study from Iran which reported the high prevalence of PMQR genes (*qnrA* and *qnrB* but not *qnrS*) in *E. coli* [36].

The remaining PMQR determinants are rarely detected in *E. coli* in the UK, as confirmed by the low detection rates of *qnrS* (1.1 %), *qepA* (1.1 %) and *oqxAB* (0.6 %) [35]. Previous studies have reported that PMQR genes are more common in *Klebsiella* species in the UK than in *E. coli*, but that they are most frequent in *E. coli* in East Asia [37]. In Algeria, *qnr* genes (A, B, S) were detected in hospital ESBLs-producing *Enterobacteriaceae* strains (CTX-M, SHV-12 and VEB-1) [27,38]. More recently, *qnr* genes were detected in *E. coli* strains producing CTX-M responsible for UTIs in Bejaia (Algeria) [33]. AmpC have been found worldwide but are less common than ESBL [39]. They are emerging worldwide in several Gram-negative bacteria as a mechanism of resistance to cefoxitin. In our study, two cefoxitin-resistant ESBL-producing isolates were carried plasmid-mediated AmpC. In Algerian and England hospitals the reported prevalence of AmpC was 1% and 7.15 %, respectively [40,41].

In our study, *E. coli* isolates were mainly groups A_0, A_1 B_1, B_2 and D_1 strains usually commensal strains, while phylogenetic groups A and B_2 strains, which are commonly extra-intestinal pathogenic were less frequent. We found also, that the prevalence of virulent genes ranged from, 4% to 26% for *sfa*. Moreover, the rate of virulence genes in Romanian uropathogenic *E. coli* strains was 86%, 36%, 23%, 14% for *fimH*, *papC, sfa/foc*, and *afa* respectively [42]. Many virulence factors facilitate *E. coli* invasion in to the vascular system causing bacteraemia and many other deep-seated infections [43].

Nosocomial urinary tract infections are responsible for significant morbidity, more predisposing factors such infections. Accurate knowledge of stem isolated bacterial during a resurgence of infections in a hospital unit, beyond the species identification and antibiotic susceptibility testing routine, has become a necessity to identify the mode of transmission of the bacteria to the host. The phenotypic usual have limitations. If a number of phenotypic traits allow define bacterial species, the expression of phenotypic traits may vary living conditions for the bacteria.

The bacterium that seems constantly feel its environment, activates or inhibits some of its genes so as to be congruent therewith. This raises a problem the reproducibility of the results can be embarrassing for a

series of comparisons strains, if one is based on phenotypic traits.

The ERIC-PCR showed the presence of 14 clones among 27 ESBL-producing *E. coli* isolates. Moreover, the fact of finding the same clone of the 2013 and 2012 strains us indicates that the situation is old and it persists, mainly in intensive care and medicine.

The sequencing of the PCR products of 27 *E. coli* strains isolated at Hospital Tlemcen Algeria helped to highlight the production of three types of ESBLs, with a clear predominance of CTX-M type ESBLs, especially CTX- M-15, or 92.59%.

These results agree with those reported by previous studies carried out in Algeria [27], Tunisia [44], Morocco [8], Nigeria [45], Mali [46] and numerous countries worldwide [47,48].

Conclusion

Our study reports the occurrence of ESBL genes in uropathogenic multidrug-resistant *E. coli* from hospitalized patients in an Algerian hospital. These isolates carried others β-lactamases and other resistance determinants such as PMQRs and 16S rRNA methylase genes. This is alarming as spread of these isolates will seriously limited options for clinical treatment in future. We hope that this phenotypical and molecular resistance data will help clinicians to better define the empiric treatment of UTIs caused by multidrug-resistant *E. coli* and to minimize the opportunity for their clonal diffusion.

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