High incidence of multidrug-resistant fecal *E. coli* producing ESBLs and carried ST131 in Jordanian adults

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Abstract

Background: *Escherichia coli* is part of the normal human intestinal microflora, although it has the potential of causing a variety of invasive and diarrheal diseases. It is also a frequent cause of community- and hospital-acquired urinary tract infections. Intestinal *E. coli* has the potential to rapidly develop multidrug resistant (MDR) and to emerge as extended-spectrum β -lactamases (ESBLs)-producer.

Methods: Over the period of July through November, 2015; 287 stool samples were collected from Jordanian adults who visited the students' clinic of the University of Jordan. Fecal samples were collected and cultured for isolation of *E. coli*. The isolates were investigated for antimicrobial susceptibility, and molecular method of polymerase chain reaction (PCR) was performed for the detection genes of ST131 clone, *bla*CTX-M group I, *bla*CTX-M-15, *bla*NDM-1, *bla*VIM, *bla*IMP, *bla*OXA-48, *bla*KPC and fluoroquinolones resistance (*gyrA* and *parC*).

Results: A total of 105/287 *E. coli* isolates (36.6%) were found to be MDR to at least 3 classes of antibiotics, of these 45.1% were ESBL-producers. A total of 51 representative MDR isolates indicated the following; 49% were found positive for ST131 clone, 58.8% were resistant for ciprofloxacin, and 41.2% were positive for CTX-M group I and CTX-M-15, respectively. All these MDR isolates were also positive for mutated both *gyrA* and *parC* genes, and only 6/51 isolates (11.8%) were positive for each *bla*NDM-1 and *bla*KPC. One out of 51 MDR isolates (2%) was positive for *bla*VIM, and none of these isolates was positive for *bla*IMP nor *bla*OXA-48 genes.

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Conclusion: This study indicated that a relatively high rates of commensal fecal *E. coli* isolates from Jordanian adults were MDR, ESBLs-producer and belonging to ST131 clone. Also, high rates of CTX-M-15 and fluoroquinolones resistance were found among MDR *E. coli* isolates.

Keywords

Fecal *E.coli* ST131; Antimicrobial Resistance; ESBLs; Jordanian Adults.

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Introduction

The increased incidence of ESBLs, that make Gram-negative bacteria frequently resistant to penicillins, cephalosporins and aztreonam by hydrolyzing them, is becoming a global health problem [1].

Class of CTX-M type enzymes are the most prevalent extended-spectrum β -lactamases (ESBLs) worldwide including Arab Middle East countries [2, 3]. Among the CTX-M type enzymes, CTX-M-15 has been frequently found in Arab countries over the last decade [3-7].

Carbapenems are part of the last line of defense against serious or invasive infections caused by extremely drug-resistant Gram-negative pathogens producing ESBLs [8-9].

Prior to the last decade, carbapenem resistance in *Enterobacteriaceae* was rare. However, *Klebsiella pneumoniae* carbapenemase (KPC), New Delhi metallo- β -lactamase-1 (NDM-1), and recently oxacillinase-48 (OXA-48) have been reported in *Enterobacteriaceae* isolates [10].

E. coli species are currently classified into four main phylogenetic groups (A, B1, B2, and D), where A and B1 groups are mainly commensal strains, whereas extraintestinal pathogenic strains are mostly part of B2 and D groups. The clone that belong to the B2 phylogenetic subgroup I, characterized by the multilocus sequence type (MLST) 131 and exhibits a specific O25 type (O25b) [11].

This new discovered ST131 clone has been recently isolated from a diverse range of infections caused in community and hospitalized patients across the world, and it has been found as ESBL CTX-M-15-producer with a high virulence potential. In addition, ST131 clone has been reported to cause major infection complications from cystitis to life-threatening sepsis in many countries [4, 12-14]. Fluoroquinolones and trimethoprim are no longer effective for empiric therapy of *E. coli* ST131 infections [13-16].

This study investigated the occurrence rate of ESBLs, especially metallo- β -lactamases and ST131 clone among *E. coli* colonized intestines of Jordanian adults.

Population and Methods

Subjects and data collection

A total of 287 feces samples using wet sterile cotton swab immersed in 3 ml 0.85% sterile normal saline tube had been collected from students (one sample from each person) whom age ranged between 18 to 35 years old visiting the University of Jordan Students' Clinic in the period from July 2015 to November 2015. Samples were transported within few hours to the microbiology research lab for culture and further investigation.

The biographic data for each student was obtained and registered on a prepared sheet which included the following: name, gender, age, university number, presence of infection and history of antibiotic treatment during the last 6 months before sampling their stool (**Table 2**).

Culture and identification of *E. coli*

All collected swabs were cultured directly on MacConkey agar and incubated for 24 hours in 37°C. Five colonies that morphologically represent *E. coli*-like growth were selected from MacConkey culture plates and sub-cultured on MacConkey again to obtain pure *E. coli* isolates. Lactose-fermenter pure growth on sub-culture plates were confirmed to be *E. coli* by standard biochemical tests, including citrate utilization, lactose and glucose fermentation on Kligler iron agar tubes, urease and indole production. All confirmed cultures of *E. coli* isolates were stored in cryotubes that contain Mueller-Hinton agar with 15% glycerol and were stored at -70 °C. *E. coli* isolates were later used for antimicrobial susceptibility test, DNA extraction and polymerase chain reaction (PCR) tests.

Antimicrobial susceptibility testing

E. coli isolates were tested using antimicrobial susceptibility disc diffusion method according to the recommendation of the Clinical Laboratory and Standards Institute (CLSI) 2015 [17]. The results were interpreted according to the breakpoints of tested antibiotics as recommended in CLSI 2015 guidelines. *E. coli* ATCC® 25922 (ATCC® is a registered trademark of the American Type Culture Collection) was used as control strain. Minimum inhibitory concentration (MIC) using E-test were used for testing MDR *E. coli* isolates (Multi-drug resistant; which are resistant to at least 3 classes of antibiotics) including resistant for ciprofloxacin and carbapenemase. The results of susceptibility tests were interpreted according to the guidelines of CLSI.

Extraction of DNA

E. coli isolates stored in cryotubes at -70°C were thawed at room temperature and cultured onto MacConkey agar. After incubation at 37°C for 24 hours, a few colonies were picked from the agar, inoculated into Mueller-Hinton broth and incubated at 37°C for 18 hours (overnight). Then, the bacterial DNA was extracted using the Wizard genomic DNA Purification Kit, Promega, (USA) following manufactures instructions. DNA extracted for each *E. coli* isolate was quantified to ensure that it is significant for PCR (Concentration > 50 ng/μL), with a purity at A260/A280=1.6-1.8, using Thermo Scientific NanoDrop 1000 Spectrophotometer.

Detection of ESBs, KPC and ST131

E. coli isolates in cryotubes stored at -70°C were thawed at room temperature, and cultured onto MacConkey agar. After incubation at 37 °C overnight few colonies were picked from the agar, and inoculated into 3ml Luria Broth (LB) and incubated for 24 hours at 37°C. The bacterial plasmid was extracted using the Zyppy™ Plasmid Miniprep Kit, Zymo (USA) according to manufacturer's instructions. Plasmid extraction was done for the identification of plasmid encoded β-lactamases genes (*bla*CTX-M group I, *bla*CTX-M-15, *bla*KPC, *bla*NDM-1, *bla*OXA-48, *bla*IMP and *bla*VIM). All Primer targets, sequences, product size and their references are shown in **Table 1**. The following controls strains were used: *K. pneumonia* (ATCC BAA-1705), *K. pneumoniae* ATCC BAA-2146; CMUL *E. coli* CL 502; CMUL *P. aeruginosa* 132, CMUL *P. aeruginosa* 123, obtained from Prof Monzer Hamza, Laboratoire Microbiologie Santé et Environnement (LMSE), Ecole Doctorale des Sciences et de Technologie, Faculté de Santé Publique, Université Libanaise, Tripoli, Lebanon).

Agarose gel preparation for gel electrophoresis

Tris Base EDTA (TBE) 1X buffer was prepared; by one part of stock solution 10X with nine parts of

Table 1. Primer targets, sequences, product sizes and references, positive control strains used in the study.

Target genes	Primer sequence	Product size(bp)	Autor	Year
CTX-M group-1	M13U (5'-GGTTAAAAAATCACTGCGTC-3') M13L (5'-TTGGTGACGATTTTAGCCGC-3')	863	Leflon-Guibout	2004
CTX-M-15	F-CTX-M-F1 (5'-ATAAAACCGGCAGCGGTG-3') R-CTX-M-F2 (5'-GAATTTGACGATCGGGG-3')	483	Leflon-Guibout	2004
ParC	F (5'-CGATTGCCGCTGAGCCACTT-3') R (5'-GCGAATAAGTTGAGGAATCAG-3')	605	Leflon-Guibout	2004
gyrA	F (5'-CGACCTTGCAGAGAGAAAT-3') R (5'-GTTCCATCAGCCCTTCAA-3')	625	Leflon-Guibout	2004
KPC	F (5'-ATGTCACGTATCGCCGTCT-3') R (5'-TTACTGCCCGTTGACGCCCA-3')	880	Pillai	2009
VIM	F (5'-GTTTGGTCGCATATCGCAAC-3') R (5'-AATGCGCAGCACCAGGATAG-3')	382	Pitout	2005
IMP	A (5'-GAAGGCGTTTATGTTTCATAC-3') B (5'-GTACGTTTCAAGAGTGATGC-3')	587	Pitout	2005
NDM-1	F (5'-GGTGCATGCCCCGTGAAATC-3') R (5'-ATGCTGGCCTTGGGGAACG-3')	660	Bonnin	2012
OXA-48	A (5'-TTGGTGGCATCGATTATCGG-3') B (5'-GAGCACTTCTTTTGTGATGGC-3')	743	Poirel	2004
trpA	F (5'-GCTACGAATCTCTGTTTGCC-3') R (5'-GCAACGCGGCCTGGCGGAAG-3')	427	Clermont	2009
pabB	F (5'-TCCAGCAGGTGCTGGATCGT-3') R (5'-GCGAAATTTTCGCCGTACTGT-3')	347	Clermont	2009

distilled water. The agarose gel at concentration of 2% was prepared by dissolving 3.6 gram of agarose powder in 180 ml of 1X buffer and boiling in microwave until agarose completely dissolved, then 8 µl of ethidium bromide (10 mg/ml) was added. The agarose was poured into the electrophoresis tray and left to solidify at room temperature for 20 minutes. After removal of combs and gates, the gel placed in the electrophoresis tank pre-filled with 1X TBE buffer to be loaded with PCR products.

Statistical analysis

Data were analyzed using Statistical Package for Social Sciences (IBM SPSS) version 21. The level of significance was set at a P value of ≤ 0.05 to test the hypothesis of no association.

Results

A total of 287 fecal samples were obtained from adult students. Of these samples, 88 were males (30.7%) with average age 21.56 ± 2.26 years and 199 (69.3%) were females with average age 21.8 ± 3.32 years (**Table 2**). The antimicrobial susceptibility pattern of 287 *E. coli* isolates is shown in **Table 3**.

Table 2. Demographic characteristics of 287 adults whose fecal samples were cultured for *E. coli* isolates*.

Characteristics	Male		Females		Total no	
	No.	%	No.	%	No.	%
Age (years)	88	30.7	199	69.3	287	100
Mean $\pm$ SD	21.58 ± 2.26		21.80 ± 3.32			
Age-range (years)	18-34		18-35			

*: The majority of students visited the clinical for treatment of respiratory tract infection. About one third of the students have stated that they have taken antibiotics during the 6 months before sampling their stools.

Table 3. Antimicrobial susceptibility patterns of 287 *E. coli* isolates*.

Antimicrobial drug	resistant isolates	
	No.	%
Ampicillin	141	49.1
Cefuroxime	139	48.4
Nalidixic acid	126	44
Tetracycline	124	43.2
Co-trimoxazole	97	33.1
Cefotaxime	46	16
Ciprofloxacin	41	14.3
Gentamicin	24	8.4
Ceftazidime	22	7.6
Imipenem	17	5.9
Amikacin	03	1

*: 105 of *E. coli* isolates (36.6%) were considered as MDR(resistant for at least 3 antibiotic classes) and 45.1% were ESBL-producers.

Table 4. Minimum inhibitory concentration range with MIC50 and MIC90 for three tested antibiotics*.

Antimicrobial drug	MIC50	MIC90	MIC Range	Breakpoints for susceptible
	µg/mL	µg/mL	µg/mL	µg/mL
Ciprofloxacin	9.9	17.8	0.002-32	≤ 0.06
Imipenem	0.1	0.17	0.002-32	≤ 1
Meropenem	0.03	0.05	0.002-32	≤ 1

*: According to CLSI 2015 guidelines.

Minimum inhibitory concentrations (MICs) of Multiple Drug Resistant (MDR) *E. coli* and the MIC ranges for three tested antibiotics (ciprofloxacin, imipenem and meropenem) are given in **Table 4**. The distribution of genes for *ESBLs*, quinolone-resistance, and ST131 clone among 51 MDR representative *E. coli* isolates are demonstrated in **Table 5**.

Sequencing results for ST131 strains

Three randomly picked positive *E. coli* ST131 isolates were sent for sequencing to Genewiz company, USA. The sequencing results were analyzed by using

Table 5. Distribution of *ESBLs* genes, quinolone-resistance, and ST131 among 51 representative MDR *E. coli* isolates*.

Specific genes	Positive <i>E. coli</i> isolates	
	No.	%
<i>bla</i> CTXM group-I	30	58.8
<i>bla</i> CTXM-15	21	41.2
<i>bla</i> KPC	6	11.8
<i>bla</i> NDM-1	6	11.8
<i>bla</i> VIM	1	2.0
<i>bla</i> IMP	Null	
<i>bla</i> OXA-48	Null	
<i>ParC</i>	51	100
<i>gyrA</i>	51	100
ST131	25	49

*: Resistant isolates to at least antibiotic classes (ampicillin, cotrimoxazole, cefuroxime).

the website <http://www.ecogene.org/gene>. Interrogation of the gene database registered sequences with identity homologies were between 98-99% for *pabB* in three isolates.

Discussion

This study investigated antibiotic resistance profiles for 287 *E. coli* isolates from fecal samples of adults visiting the students' clinic of the University of Jordan. The study found that 36.6% (105) of these students carried MDR fecal *E. coli* for at least 3 classes of antibiotics. *E. coli* ESBL-producers were found to be 45.1% among 105 MDR tested isolates. These results are similar to two recent studies examined fecal samples from hospitalized and out-patient infants admitted to the Pediatric Clinic at JUH during the years 2011 to 2014, and both studies reported rates of 30.6% and 42% MDR *E. coli* isolates which were ESBL-producers [4, 6].

The emergence of antimicrobial resistance is becoming a serious global health problem worldwide, especially among pathogenic *E. coli* [1, 23]. Most

published data on antimicrobial resistance were obtained from hospitalized or out-patients. Few studies have investigated the occurrence of antimicrobial resistance in a community population. Healthy individuals may carry MDR fecal *E. coli*, and these individuals can spread their resistant *E. coli* in their community, and might be a potential source of community infections [24].

A recent study, which was carried at the same period in four medical centers in Jordan and Lebanon over the period between 2011 and 2013, and included samples from patients have UTI and intra-abdominal infection (IAI), has shown that ESBL-producers among *E. coli* isolates were 43% and 49% in UTI and IAI samples, respectively [26]. A Libyan study investigated 333 *Enterobacteriaceae* isolates from both patients and healthy controls, and found only 15 (4.5%) isolates were positive for ESBL using the double disc synergy test. The Libyan study also detected that patients were infected with 5.4% of *E. coli* and 11.4% of *K. pneumoniae* isolates which were ESBL-producers. Additionally, the study indicated that almost all the ESBL-producing isolates were consistently more resistant to a wide variety of antimicrobial agents than the non-ESBL-producers as it is the case in our study [27]. The low incidence of ESBL-producers in the Libyan study may be due to use a detection test which is not sensitive enough like molecular detection using PCR which was applied in our study and often in other studies. Other recent studies from the Arab Middle East region documented that rates of *E. coli* ESBL-producers remain higher in clinical samples when compared to healthy persons [5, 28].

This study showed a relatively high percentage of blaCTX-M group I and blaCTX-M-15 among the MDR *E. coli* isolates (58.8% and 41.2%, respectively). It has been previously documented that blaCTX-M-15 group is widely prevalent in Arab Middle East region [3-7]. Currently, blaCTX-M-15 type is commonly detected in ESBL positive *Enterobacteria-*

ceae and others Gram-negative pathogens through the whole world [2, 29].

This study also demonstrated presence of different β -lactamases other than CTX-M types such as KPC, NDM-1 which had been detected in 11.8%, and VIM in 2% of MDR isolates, while no detection of IMP and OXA-48 was recognized (**Table 5**). These results are novel, since no previous study in Jordan has reported on the occurrence of KPC, NDM-1 β -lactamases in *E. coli* isolates from any source.

This study detected *ParC* and *gyrA* quinolone-resistance genes among all 51 MDR *E. coli* isolates. This high percentage may indicate extensive consumption of fluoroquinolones in Jordan according to our available information. Another suspected factor which may contribute to high fluoroquinolone-resistance is the dissemination of ST131 *E. coli* clone [4, 30]. The present study shows that *E. coli* ST131 clone carrying the *pabB* gene and ciprofloxacin-resistant was accounted for 49% of MDR *E. coli* isolates. This result is relatively high when compared to a recent study investigated fecal *E. coli* isolates from Jordanian infants admitted to the JUH, and found only 9 isolates (7.2%) to be positive for ST131 clone [4]. A study in US found that ST131 accounted for 46% of ESBL-positive *E. coli* isolates [30]. Other studies from developed countries reported on the emergence of *E. coli* ST131 types in geriatric patients and among hospitalized patients colonized intestinally with fluoroquinolone-resistant *E. coli*. [30-32]. In addition, a recent paper from Japan, found Type 131 C1-M27 sub-clone, among ESBL-Producing *E. coli* in wastewater [33].

This study suggests that continuous increasing incidence of *E. coli* producing ESBL and fluoroquinolone-resistance in intestinal tract of population would make the treatment of infections caused by *E. coli*, especially community UTIs more difficult and challenging.

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Ethical considerations

This study has been approved by the Faculty of Medicine and the Faculty of Graduate studies, University of Jordan. Also, permission was obtained from the Institutional Review Board (IRB) and Ethical Committee at Jordan University Hospital (Permission No. 22/2015)

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