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Detection of *Escherichia coli* O_{157}:H_7 and O_{104}:H_4 in patients with diarrhea in Northern Lebanon and characterization of fecal *E. coli* producing ESBL and carbapenemase genes

Sara Amrieh[1], Monzer Hamze[1,2]*, Hassan Mallat[1,2], Marcel Achkar[3], Fouad Dabboussi[1,2]

1 Health and Environmental Microbiology Laboratory (LMSE), AZM Center for Research in Biotechnology, Doctoral School of Science and Technology, Lebanese University, Tripoli - Lebanon.
2 Faculty of Public Health, Lebanese University, Tripoli - Lebanon.
3 NINI Hospital Laboratory, Tripoli - Lebanon

Corresponding author:

Prof. Dr. Monzer HAMZE

mhamze@monzerhamze.com

Abstract

Background. While most strains of *Escherichia coli* (*E. coli*) are harmless, some are causing intestinal infections of varying severity. The Shiga toxin-producing *E. coli* (STEC)/ enterohemorrhagic *E. coli* strains can be associated with fatal clinical manifestations. Of these *E. coli* Serotypes O_{157}: H_7 and O_{104}: H_4 were responsible for worldwide epidemics causing thousands of intestinal infections and dozens of deaths. The aim of this research is to investigate the prevalence of *E. coli* O_{157}: H_7 and O_{104}: H_4 in the diarrheal stools of 242 Lebanese patients.

Materials and methods. This study includes 242 *E. coli* strains isolated from fecal specimens of patients with diarrhea between February 2013 and May 2014 in the microbiology department of Nini Hospital Laboratory in Tripoli - North Lebanon. All specimens were inoculated on sorbitol MacConkey agar. Sorbitol negative strains were investigated for detection of *stx1*, *stx2* and *eae* genes using real-time PCR. All carbapenem-resistant strains and ESBL producers were investigated by PCR for presence of KPC, IMI, NMC-A, EMS, GHG, VIM, NDM, IMP, OXA-48, bla_{TEM}, bla_{CTX-M}, bla_{SHV}, bla_{OXA}, bla_{GES} and bla_{PER}..

Results. A total of 14 sorbitol negative strains were detected. The search for *stx1*, *stx2* and *eae* genes showed the presence of a single positive strain for *E. coli* O_{157}: H_7. Out of 242 *E.coli* strains, 48 (19.8%) were ESBL-positive, 4 (1.6%) were resistant to ertapenem, and all were negative for *stx2* genes, The bla_{CTX-M} gene was the most frequent

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among ESBL positive strains (85%), followed by the bla_{TEM} gene (50%). One strain had the bla_{NDM-1} gene, another had the bla_{OXA-48} gene and 2 strains were probably resistant due to impermeability.

Conclusion. The results of this study demonstrate rarely presence of enterohemorrhagic *E. coli*, but shows the frequent presence of multi-drug resistant *E.coli* in the intestinal flora of North Lebanese patients. Therefore, it is important to search for MDR *E.coli* in the intestinal flora of patients who are going to be treated with major operations or those admitted to intensive care units.

Keywords: *E. coli* O_{157}:H_7, *E. coli* O_{104}:H_4, resistance to carbapenems.

Introduction

There are about 1.7 billion cases of diarrhea each year worldwide. It was considered the second cause of mortality in children under five years of age and was responsible for 760.000 child deaths per year according the report of WHO in 2013 [1]. Rotavirus and *E. coli* are the two most common causes of diarrhea in developing countries [1]. The majority of *E. coli* strains are commensal organisms, however, some were associated with intestinal and extra-intestinal diseases. Among these, the Shiga toxins producing *E. coli* (STEC)/enterohemorrhagic *E. coli* (EHEC) strains which are a major cause of bacterial gastroenteritis in the world [2]. There are six pathotypes of intestinal *E. coli* described in literature, associated with acute diarrheal disease on the basis of their expressed virulence factors; ETEC "enterotoxigenic *E. coli*," EPEC "enteropathogenic *E coli,* "EHEC" enterohaemorrhagic *E. coli,* "EIEC" enteroinvasive *E. coli,* "EAggEC" enteroaggregative *E. coli,* and "DAEC" diffusely adhering *E. coli* [3]. Infection with an EHEC strains can take various clinical forms ranging from asymptomatic carriage to fatal infection. The most common clinical manifestation is Hemorrhagic Colitis (HC) which can be progressed to Hemolytic Uremic Syndrome (HUS), or Thrombotic Thrombocytopenic Purpura (TTP) especially in children and the elderly patients[4]. EHEC O_{157}: H_7 is the serotype most important to public health but other serotypes have been frequently involved in sporadic cases and outbreaks [5, 6]. During May- June 2011, a large outbreak of *E. coli* enteroaggregative Hemorrhagic (EAHEC) O_{104}: H_4 was reported in Germany and led to HUS in more than 800 patients, many of whom were adults, and ultimately resulted in 54 deaths [6, 7]. The severity of this outbreak was significantly higher due to the serotype O_{104}: H_4 with over 20% cases of HUS and 1.4% of deaths than with other the serotype O_{157}: H_7. Those HUS cases were affected mainly young children with a mortality rate of 0.5% [8]. While it

is recognized that commensal fecal bacteria were important in the emergence of resistance under antibiotic selection pressure. Recently, the main threat is the spread of bacteria with resistant to carbapenems in intestinal of people, since carbapenems are currently the only cure for many serious infections caused by multidrug-resistant (MDR) bacteria [9].

This research aimed to investigate the prevalence of *E. coli* O_{157} and *E. coli* O_{104} in stools of patients with diarrhea in Northern Lebanon by detection their virulence genes *stx1*, *stx2* and *eae* using real time PCR . Also at the same time to evaluate the rate of fecal *E.coli* isolates producing ESBL and carbapenems- resistant.

Materials and methods

Place and period of the study

This study carried out over the period March 25, 2014 and August 8, 2014 in the microbiology laboratory, health and environment, at the Azm research center for biotechnology which is part of the graduate doctoral school of the Lebanese University.

Fecal E.coli isolates

A total of 242 fecal specimens of diarrheal patients were sent to Microbiology Department of Nini Hospital Laboratory in Tripoli - North Lebanon. All specimens were cultured on sorbitol MacConkey agar (Conda®-Spain); the culture plates were incubated for 24 hours at 37 °C. All *E.coli* isolates were identified by the Api 20E gallery (Biomérieux®-France) according to the protocol suggested by the manufacturer.

Screening for EHEC O157: H7 strains

All sorbitol negative *E.coli* isolates were investigated for *stx1*, *stx2* and *eae* genes using real-time PCR utilizing ready to use kits ("Foodproof® STEC Screening LyoKit"(BIOTECON Diagnostics Gmbh – Germany).

Determination of positive ESBL strains and/or resistant to carbapenems

For the detection of ESBL type resistance and/or the presence of carbapenems resistance, we followed the recommendations of the Antibiogram Committee of the French Society of Microbiology - 2014 (CA-SFM, 2014) [10]. For each strain showing resistance to ertapenem or an ESBL profile using antibiotic susceptibility by the disc method on Muller-Hinton agar (Biorad®, France) according to the recommendations of the CA-SFM -2014. [10] Finally, the Hodge test was performed for strains resistant to ertapenem [11].

Determination of resistance by production of cephalosporinase.

The inhibition tests are based on the increase in the diameter of inhibition around a disc combining a carbapenem (meropenem or imipenem) and a specific inhibitor of β-lactamases. By testing bacteria producing beta-lactams on a medium containing cloxacillin (250 mg/l) (cephalosporinase inhibitor) and comparatively on a medium without cloxacillin, we can detect a resistance to carbapenems not related to the production of carbapenemase but to the association of cephalosporinase and by default the accumulation of carbapenems which results in a significant increase of the inhibition diameters on the first medium .

Molecular Study of carbapenems resistant strains

A total of 4 *E.coli* carbapenem-resistance isolates were examined for presence the genes of KPC, IMI, NMC-A, EMS, GHG, VIM, NDM, IMP and OXA-48 (**Table 1)**. Each isolate is cultivated on nutrient agar (Biorad®, France) and incubated for 24 hours at 37°C. Few colonies of each isolates were suspended in 0.5 ml of ultrapure sterile water, their DNA were extraction using the kit "GenEluteTM Bacterial Genomic DNA Kit, *Sigma-Aldrich*.".and stored at -20 °C. The PCR was performed using the following

Table 1. The different primer sequences for each carbapenemase and ESBL gene and the PCR corresponding conditions.

Genes	Sequence	Amplicon size (pb)	Reference
bla$_{KPC}$ F *bla*$_{KPC}$ R	ATGTCACTGTATCGCCGTCT TTACTGCCCGTTGACGCCC	882	[12]
bla$_{SME}$ F *bla*$_{SME}$ R	ACTTTGATGGGAGGATTGGC ACGAATTCGAGCATCACCAG	551	[13]
bla$_{IMI-NMC}$ F *bla*$_{IMI-NMC}$ R	TGCGGTCGATTGGAGATAAA CGATTCTTGAAGCTTCTGCG	399	[13]
bla$_{GES}$ F *bla*$_{GES}$ R	CTATTACTGGCAGGGATCG CCTCTCAATGGTGTGGGT	594	[14]
bla$_{VIM}$ F *bla*$_{VIM}$ R	GATGGTGTTTGGTCGCATA CGAATGCGCAGCACCAG	170	[15]
bla$_{IMP}$ F *bla*$_{IMP}$ R	GAGTGGCTTAATTCTCRATC AACTAYCCAATAYRTAAC	120	[14]
bla$_{NDM-1}$F *bla*$_{NDM-1}$R	GGTGCATGCCCGGTGAAATC ATGCTGGCCTTGGGGAACG	660	[16]
bla$_{OXA-48}$ F *bla*$_{OXA-48}$ R	GGGGACGTTATGCGTGTATT GAGCACTTCTTTTGTGATGGC	900	[17]
bla$_{TEM}$-F *bla*$_{TEM}$-R	ATGAGTATTCAACATTTCCG CTGACAGTTACCAATGCTTA	867	[18]
bla$_{SHV}$-F *bla*$_{SHV}$-R	GGTTATGCGTTATATTCGCC TTAGCGTTGCCAGTGCTC	867	[18]
bla$_{OXA}$-F *bla*$_{OXA}$-R	ACACAATACATATCAACTTCGC AGTGTGTTTAGAATGGTGATC	885	[18]
bla$_{CTX-M}$U1 *bla*$_{CTX-M}$U2	ATGTGCAGYACCAGTAARGT TGGGTRAARTARGTSACCAGA	593	[18]
bla$_{GES}$-A *bla*$_{GES}$-B	CTTCATTCACGCACTATTAC TAACTTGACCGACAGAGG	827	[18]
bla$_{PER}$-A *bla*$_{PER}$-B	GGGACARTCSKATGAATGTCA GGGYSGCTTAGATAGTGCTGAT	827	[18]

protocol: 12.5µl Mix (REDTaq® ReadyMixTM PCR reaction mix with MgCl2. *Sigma – Aldrich* - Germany) was mixed with 5.5 µl of ultrapure water, 5 µl of plasmid extract, and 2 µl of each primer. The mixture was incubated in a thermo cycler. PCR products were analyzed by electrophoresis in 1% agarose gel to visualize the amplicon bands .

Molecular studies of ESBL strains

A total of 48 *E.coli* producing ESBL isolates were tested for the following beta-lactamse genes: *bla*$_{TEM}$, *bla*$_{CTX-M}$, *bla*$_{SHV}$, *bla*$_{OXA}$, *bla*$_{GES}$ and *bla*$_{PER}$ (**Table 1**). The same protocol described above was applied.

Table 2. Antibiotics sensitivity profile of ESBL and carbapenems resistant strains

		Carbapenems resistant strains			
Antibiotic	**% of Sensitivity (ESBL strains)**	**1**	**2**	**3**	**4**
Cefoxitin	81.2	R	R	R	R
Cefepime	8.3	I	R	R	R
Piperacillin-Tazobactam	79.1	R	R	R	R
Ticarcillin-Clavulanate	52.0	R	R	R	R
Cefotaxime	0	R	R	R	R
Amoxicillin-Clavulanate	62.5	R	R	R	R
Ceftazidime	0	R	R	R	R
Ticarcillin	2.0	R	R	R	R
Cefixime	0	R	R	R	R
Aztreonam	2.0	I	R	R	R
Cefuroxime	0	R	R	R	R
Piperacillin	0	R	R	R	R
Ertapenem	95.8	R	R	R	R
Imipenem	97.9	S	R	S	R
Meropenem	95.8	S	R	S	R
Ampicillin	0	R	R	R	R
Amikacin	97.9	S	S	S	S
Netilmycin	77.0	R	S	S	I
Colistin	100	S	S	S	S
Trimetroprim-Sulfamtoxazole	37.5	R	R	R	R
Tobramycin	60.4	R	R	R	R
Gentamicin	66.6	R	S	R	R
Ofloxacin	58.3	R	R	R	R
Ciprofloxacin	62.5	R	R	R	R
Nalidixic acid	27.0	R	R	R	R
Tigecyclin	97.9	S	S	S	S
Minocyclin	77.0	S	R	R	S
Tetracyclin	43.7	S	R	R	R
Fosfomycin	97.9	S	S	S	S

Detection of EHEC O_{104} strains among ESBL positive strains

Positive isolates for *Stx2 (lp43, lp44)* gene was detected using conventional PCR for ESBL positive strains according to Bielaszewska *et al.* [6].

Results

Out 242 of *E. coli* isolates, 14 strains were sorbitol negative (5.78%). Of these only one isolate was positive for *stx1*, *stx2* and *eae* genes. This isolate was isolated from a twenty year-old girl hospitalized for severe diarrhea and the search for presence of *Salmonella, Shigella* and the Rotavirus antigen was negative. The isolate was susceptible to all antibiotics routinely tested for enteric bacteria in the laboratory.

All ESBL-positive *E. coli* isolates were negative for *stx 2* gene (**Table 2**).

The distribution of ESBL 6 genes (bla_{TEM}, bla_{CTX-M}, bla_{SHV}, bla_{OXA}, bla_{GES} and bla_{PER}) are shown in **Table 3**.

The resistance profile of the carbapenems-resistant *E.coli* isolates is shown in Ttable 2. Of the 4 isolates resistant to ertapenem, 2 were positive for the Hodge test, one had the bla_{OXA-48} gene and the

Table 3. Results of ESBL genes characterization

Genes types	No (%) Strains
bla_{TEM}	24 (50)
bla_{CTX-M}	41 (85.4)
bla_{OXA}	3 (6.2)
bla_{SHV}	0
bla_{PER}	0
bla_{GES}	0
bla_{TEM}-bla_{CTX-M}	17 (35.4)
bla_{OXA}-bla_{CTX-M}	3 (6.2)

other had the *bla*$_{NDM-1}$ gene. The other 2 isolates were negative for the Hodge test and negative for all the ESBL genes.

Discussion

According to the surveillance unit of enteric communicable diseases in Lebanon, a report issued in 2012 showed high occurrence rate of typhoid fever (426cases). The report also observed high incidence of food poisoning (319 cases) and 176 dysentery cases [19]. The prevalence of *E. coli* O_{157}: H_7 and *E. coli* O_{104}: H_4 among diarrheagenic cases is still unknown in Northern Lebanon. Stool cultures are routinely not culture to detect enterohaemorrhagic *E. coli* in Lebanon, and searching for this organism is only done if requested by the physician. Our results showed a rare presence of *E. coli* O_{157}: H_7 (0.4%), (one isolates out 242 diarrhea cases), and there is no single *E. coli* O_{104} isolate. A similar result was reported recently in Jordan [20]. In Iraq, one study reported a prevalence of 11.5% for EHEC O_{157}: H_7 in the stool of 200 children with hemorrhagic diarrhea [21]. About 93% of *E. coli* strains of human origin ferment sorbitol in 24 hours; conversely *E. coli* O_{157} does not ferment sorbitol [22], but other study reported that some STEC O_{157} strains may ferment sorbitol within 24 hours [23]. The emergence of the epidemic strain EAHEC O_{104}: H_4 depends on the acquisition of prophage *stx2* and a plasmid encoding ESBL (CTX-M-15) [24]. That is why we looked for the presence of the *stx 2* gene in all ESBL positive *E. coli*, the results were negative. The EAHEC O_{104}: H_4 has caused a major epidemic of diarrhea cases during 2011 in Germany, where 3842 patients infected and 855 cases developed HUS [7]. Another small outbreak was reported in France during June 2011 [25].

The present study shows that 19.8% of *E.coli* isolates from fecal specimens of Lebanese patients with diarrhea were ESBL-producers .This result is similar to other recent reported studies from other continents . For example a study from Argentina in 2012, showed that the carrier rate of ESBL-positive *E. coli* strains in the fecal flora was 17.0% [26], while the study from Libya in 2014, demonstrated the prevalence of ESBL-producing *E. coli* at 13.4% [27]. In addition, our study indicated that *bla*$_{CTX-M}$ gene was predominant (85%), followed by the *bla*$_{TEM}$ (50%) and *bla*$_{OXA}$ (6%) genes, whereas the *bla*$_{SHV}$, *bla*$_{PER}$ and *bla*$_{GES}$ were absent in our *E.coli* isolates. A French study showed a predominance of the *bla*$_{CTX-M}$ gene (86%) followed by the *bla*$_{SHV}$ gene in *E. coli* strains isolated from fecal flora (14%) [28], while a Spanish study detected less rate of ESBL-producing *E. coli* in stool samples of healthy males from in 2007 [29].

It is important to note that almost all ESBL-producing *E.coli* isolates were susceptible to amikacin (97.9%), to less extent to cefoxitin and piperacillin-tazobactam combination, and with high rate of resistance to fluoroquinolones (Table 2). In Egypt, a study reported in 2009, that ESBL-positive *E. coli* strains isolated from the fecal flora have resistance rate of 39.4% to fluoroquinolones and 11.9% to amikacin, while all were susceptible to carbapenems [30]. A Jordanian study reported recently that MDR *E.coli* isolates from feces of infants accounted for 30.6%, and all were ESBL producers. The detection rate of CTX-M genes among these isolates was 94.2%, and CTX-M group 1 accounted for 87.8% of the isolates, and 73.2% were CTX-M-15 producers [31]. This study shows that few *E.coli* isolates (4) were resistant to ertapenem, including one strain carried *bla*$_{NDM-1}$ gene and another strain has the *bla*$_{OXA-48}$ gene. A previous study by Beyrouthy *et al.* in Lebanon reported on the prevalence of carbapenems-resistant among *Enterobacteriaceae* in the fecal flora of healthy children. The study showed that 3/183 investigated *E.coli* strains were resistant to ertapenem, all carried *bla*$_{OXA-48}$ gene, and their study suggests that *bla*$_{OXA-48}$ has become endemic in Northern Lebanon [32]. In China, the prevalence of

carbapenems-resistant among *Enterobacteriaceae* in the fecal flora of hospitalized patients was 6.6% (20/303 patients), of which 8 isolates were carried bla_{KPC-2}, bla_{IMP-4} and bla_{NDM-1} and typed as carbapenemase producers [33]. Another study conducted in South Korea, showed that the carrier rate of carbapenems-resistant fecal *E.coli* strains was 1.44% (5/347) [34].

In conclusion, our results shows the importance of investigation continuously the fecal stools of patients for prevalence of MDR *E.coli* and other enteric bacteria, particularly it is necessary to characterize the ESBL type resistance genes including gene sequencing of carbapenems and perform molecular epidemiological studies by pulsed field gel electrophoresis.

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