Original Article

Molecular characterization of ESBL-producing *Shigella sonnei* isolates from patients with bacilliary dysentery in Lebanon

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Abstract

Background: Emergence of extended-spectrum β-lactamases (ESBLs) in *Shigella* species imparting resistance to third-generation cephalosporins is a growing concern worldwide. The aim of this study is to molecularly characterize the newly emerging beta-lactam resistant *Shigella sonnei*, specifically ESBLs in Lebanon, and compare them to beta-lactam sensitive isolates.

Methodology: We compared five beta-lactam-resistant *S. sonnei* isolates to six isolates susceptible to beta-lactams. Presence of ESBLs was established by the combination disk method. PCR amplification and sequence analysis of the beta-lactamase-encoding genes, along with other antimicrobial resistance genes, were performed. The localization of beta-lactamase genes was established by conjugation experiments. Beta-lactamase gene transcription levels were determined by real-time RT-PCR. Molecular typing was performed by pulsed-field gel electrophoresis (PFGE).

Results: Four of five beta-lactam resistant isolates were extended spectrum beta-lactamase producers. These harbored the *bla*-$_{\text{CTX-M-15}}$ gene borne on a 70 Kb plasmid and class 2 integron genes on their chromosomes. The *bla*-$_{\text{CTX-M-15}}$ gene was flanked by an insertion element IS*Ecp1*. A chromosomal *bla*-$_{\text{TEM-1}}$ gene was detected in one beta-lactam resistant *Shigella* isolate and two of the ESBL producing isolates. The *bla*-$_{\text{CTX-M-15}}$ gene transcription levels were increased in EBSL isolates exposed to subinhibitory concentrations of ceftazidime. PFGE analysis revealed that the four *bla*-$_{\text{CTX-M-15}}$ positive isolates were nonclonal but two of them shared genotypes with β-lactam susceptible isolates.

Conclusion: Dissemination of broad-spectrum beta-lactam resistance in *Shigella sonnei* is mediated by *bla*-$_{\text{CTX-M-15}}$ through horizontal plasmid transfer rather than by clonal spread of the resistant isolates. Expression of this gene is further induced in the presence of ceftazidime.

Keywords: *Shigella sonnei*, extended spectrum beta-lactamase, CTX-M-15, pulsed field gel electrophoresis, gene expression

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Introduction

In recent years, increased incidence of resistance to beta-lactams among members of the family *Enterobacteriaceae* has been reported worldwide [1]. ESBL-producing gram-negative bacteria are becoming a major global concern and usually harbor plasmid-mediated enzymes of the TEM, SHV, OXA, PER, and CTX-M types [1-3].

Shigella species have been progressively acquiring resistance to several antimicrobial agents used for the treatment of infections with these bacteria [4]. Several reports have indicated an increase in cases of *Shigella* species resistant to beta-lactams, including third-generation cephalosporins [5]. So far only a few cases or small case series of ESBL-producing *Shigella* have been reported from certain countries including Argentina [6,7], Hong Kong [8], Turkey [9,10], Korea [1], Bangladesh [11], and Israel [12].

Previous studies conducted in Lebanon on various species of the *Enterobacteriaceae* showed the prevalence of the TEM- and SHV-type ESBL-producing *E. coli* [13] and of CTX-M-15 type ESBL [14,19]. Recently, three ESBL-producing *Shigella sonnei* isolates were isolated for the first time in Lebanon from patients with shigellosis at a tertiary medical center in Beirut [15]. Two additional beta-lactam resistant *S. sonnei* isolates were encountered, one of which harbored an ESBL. This prompted us to further characterize all ESBL-producing clinical isolates of *S. sonnei* at the molecular level, in comparison with beta-lactam susceptible isolates.

Materials and Methods

Source and Identification

Sixty *S. sonnei* isolates were collected from stool samples of different patients admitted for bacilliary dysentery to a tertiary care center in Beirut, Lebanon, between July 2004 and June 2007**.** Antimicrobial susceptibility testing was performed using a panel of antimicrobials, according to CLSI guidelines [16]. ESBL production was established when an increase of ≥ 5 mm of the inhibition zone was observed around the cetazidime/cefotaxime disks with clavulanic acid versus ceftazidime/cefotaxime alone. Eleven of the 60 isolates were selected for the study, namely the five beta-lactam resistant isolates and six randomly selected control beta-lactam susceptible isolates (Table 1).

Table 1: Patient data, antimicrobial susceptibility profiles and molecular results for beta-lactamase genes in 11 *Shigella sonnei* isolates.

Shigella sonnei **Isolate**	**Age/Sex**	**Antimicrobial Susceptibility Profile**								**β-Lactamase Encoding genes**	
		AMP	**CTX**	**CAZ**	**CTR**	**CIP**	**NOR**	**SXT**	**CAZ/CLA**	***bla*$_{\text{-CTX-M-15}}$**	***bla*$_{\text{-TEM-1}}$**
1	9/F	R	R	S	R	S	S	R	S	+	+
2	41/F	R	S	S	S	S	S	R	S	-	+
3	5/M	R	R	S	R	S	S	R	S	+	+
4	3/M	R	R	I	R	S	S	R	S	+	-
5	7/M	R	R	I	R	S	S	R	S	+	-
6	36/F	S	S	S	S	S	S	R	ND	-	-
7	24/M	S	S	S	S	S	S	R	ND	-	-
8	3/F	S	S	S	S	S	S	R	ND	-	-
9	1/M	S	S	S	S	S	S	R	ND	-	-
10	10/M	S	S	S	S	S	S	R	ND	-	-
11	40/F	S	S	S	S	S	S	R	ND	-	-

AMP: Ampicillin, CTX: Cefotaxime, CAZ: Ceftazidime, CTR: Ceftriaxone, CIP: Ciprofloxacin, NOR: Norfloxacin, SXT:Trimethoprime/sulfamethoxazole, CAZ/CLA: Ceftazidime-clavulanic acid; S= sensitive; R= resistant; I= intermediate; ND= Not done; (+)= positive; (-)= negative; (M)= male; (F)= female

Total and Plasmid DNA extractions

A total of 11 *S. sonnei* clinical isolates were subjected to total DNA extraction using a GFX^{TM} Genomic Blood Purification Kit (Amersham Pharmacia Biotech, Piscataway, NJ). Plasmid DNA was extracted using the Plasmid DNA Purification kit (NucleoSpin® Plasmid QuickPure, Macherey-Nagel, Germany).

Polymerase Chain Reaction

Polymerase chain reaction (PCR) was performed on both the total and plasmid DNAs of the 11 *S. sonnei* isolates as previously described [15] using a PTC-100 thermal cycler (MJ Research Inc., Watertown, MA). PCR targets included three beta-lactamase-encoding gene categories (*bla*$_{\text{-CTX-M}}$, *bla*$_{\text{-SHV}}$ and *bla*$_{\text{-TEM}}$) in addition to the genes encoding fluoroquinolone-modifying enzymes: *qnrA*, *qnrB*, *qnrS*, class I and II integrons, *aac (6')-Ib-cr*, *oxa-1*, and the IS*Ecp1B* insertion sequence [15]. PCR products were detected on 1.5 % agarose gel (FMC Bioproduct, Rockland, ME) after ethidium bromide staining and UV illumination, photographed with an Olympus digital camera and analyzed using the Digi-Doc-it software (UVP, Upland, CA).

Sequence analysis

Unincorporated dNTPs and primers were removed from PCR amplicons of the five beta-lactam resistant *S. sonnei* isolates using a QIAquick PCR purification kit (QIAGEN, Valencia, CA). Sequencing was performed using the BigDye 3.1 Terminator kit. Purified sequencing products were analyzed on the ABI 3100 genetic analyzer (Applied Biosystems, Foster City, CA). Sequence data were then analysed using the Seqman software package (DNASTAR, Madison, WI).

Conjugation

Conjugation experiments were performed using the beta-lactamase positive *S. sonnei* isolates as parents and *E. coli* J53 (sodium azide-R) as the recipient on MacConkey agar containing 100 mg/L sodium azide plus 2.0 mg/L cefotaxime [15]. *E. coli* 39R861 (NCTC 50192) and *E. coli* V417 (NCTC 50193) were employed for sizing of extracted plasmids after electrophoresis through 1.5% agarose gels. Plasmid-encoded *bla*$_{\text{CTX-M-15}}$ gene was detected by PCR from both the parents and transconjugant strains.

Real time PCR (RT-PCR)

Bacteria were incubated with serial dilutions of ceftazidime. The highest dilution with bacterial growth and the positive control (bacteria without ceftazidime) were used as follows: 1 ml of bacterial suspensions adjusted to 0.5 MacFarland standard were subjected to RNA extraction with the RNA spin mini kit (GE Healthcare). Reverse transcription was conducted on extracted RNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics GmbH, Mannheim, Germany). Real-time PCR of the c-DNAs was performed using two sets of primers yielding a 200 bp product targeting the *bla*$_{\text{-CTX-M-15}}$ and the housekeeping GlgC gene (Glucose-1-phosphate adenylyltransferase gene) (Table 2). The 20 µl reaction was performed on the Light Cycler® 2.0 (Roche Applied Science, GmbH, Mannheim, Germany) using standard cycling parameters. It contained 10 µl of ABsolute SYBR Capillary Mix (ABgene, UK), 2 µl of each primer (0.5µM each), 2 µl of c-DNA and 4 µl of molecular grade water. Relative quantification ratios reflecting relative gene expression of *bla*$_{\text{-CTX-M-15}}$/GlgC were generated using the LightCycler® Software version 4.05 (Roche Diagnostics GmbH, Mannheim, Germany).

Pulsed Field Gel Electrophoresis

Pulsed field gel electrophoresis (PFGE) of macrorestricted chromosomal DNA on 11 isolates was performed as previously described using the PULSENET USA standard *Salmonella enterica* serovar Braenderup strain H9812 as a molecular size marker (www.cdc.gov/PULSENET). Analysis of the PFGE gel image was performed with GelComparII version 5.1 (Applied Maths, Sint Martin Latem, Belgium).

Results

Four of five *S. sonnei* isolates resistant to beta-lactams were ESBL producers (isolates 1, 3, 4, and 5, Table 1). Another beta-lactam non-ESBL producing isolate was resistant to ampicillin (isolate 2, Table 1). The remaining 55 isolates were susceptible to beta-lactams. Eleven isolates were selected for further study including all 5 beta-lactam resistant isolates. These were obtained from five male and six female patients with ages ranging between 1 and 41. All *Shigella* isolates were susceptible to quinolones (Table 1).

PCR amplification of genomic DNA extracted from the five beta-lactam resistant *S. sonnei* isolates showed that all four ESBLs producing isolates harbored a *bla*-$_{\text{CTX-M}}$ encoding gene. Two of these isolates, as well as the one beta-lactam resistant ESBL-negative isolate, harbored a *bla*-$_{\text{TEM}}$ encoding gene (Table 1). PCR amplification of the *bla*-$_{\text{CTX-M}}$ and *bla*-$_{\text{TEM}}$ genes from the plasmid DNA of the five beta-lactam resistant *S. sonnei* isolates showed the presence of only the *bla*-$_{\text{CTX-M}}$ encoding gene in all four ESBL isolates (Table 1). This indicates that the *bla*-$_{\text{TEM}}$ gene is chromosomal while the *bla*-$_{\text{CTX-M}}$ gene is plasmid-encoded. Furthermore, sequence analysis demonstrated that the recovered *bla*-$_{\text{CTX-M}}$ gene in each of the four isolates was *bla*-$_{\text{CTX-M-15}}$ and that the *bla*-$_{\text{TEM}}$ encoding gene in all three positive isolates was the *bla*-$_{\text{TEM-1}}$ gene. Six beta-lactam-susceptible isolates were PCR negative for the beta-lactamase encoding genes. None of the *S. sonnei* isolates were found to harbor a *bla*-$_{\text{SHV}}$ encoding gene or the fluoroquinolone resistance genes [*qnrA, qnrB, qnrS,oxa1* or *aac*(6')-Ib-cr].

Table 2: Oligonucleotides used for Real time RT-PCR amplification of the *bla*$_{\text{-CTX-M-15}}$ gene and the housekeeping GlgC gene

PCR target	Primer name	Primer sequence	Product size (bp)
bla$_{\text{-CTX-M-15}}$ gene	CTX-M-15-F	5'-TAAAGCATTGGGCGACAG-3'	200
	CTX-M-15-R	5'-GGTGAAGTAAGTGACAATC-3'	
GlgC	GlgC-F	5'-ATGAGTATTGAACATTTCCG-3'	200
	GlgC-R	5'CCAATGCTTAATCAGTGAGG-3'	

Conjugation experiments showed the transfer of the plasmid encoded *bla*-$_{\text{CTX-M-15}}$ gene from the four ESBL producing *S. sonnei* isolates to J53 *E. coli*. Transconjugants demonstrated resistance to third-generation cephalosporins mediated by transfer of a 70 kb plasmid. The presence of a *bla*-$_{\text{CTX-M}}$ gene on the plasmid DNA of the transconjugants was determined by PCR (data not shown). The *bla*-$_{\text{CTX-M-15}}$ genes recovered in four ESBL-producing isolates were flanked by the mobilizing insertion sequence IS*Ecp1*. Although class-I integrons were not detected, class-II integrons (all containing *dhfr1*, *aadA1* and *sat1*) were present in the parents but not

in the transconjugants and must therefore be chromosomally encoded.

We used real-time RT-PCR to perform relative quantification of expression of the *bla*-$_{CTX-M-15}$ gene as compared to the housekeeping *glgC* gene in the presence of ceftazidime subinhibitory concentration (Table 3). The level of expression of the beta-lactamase gene was increased two- to six-fold in three tested ESBL-positive isolates.

PFGE analysis of XbaI-restricted genomic DNA from the 11 *S. sonnei* isolates revealed the presence of six distinct patterns that included two clusters of three and four isolates, respectively displaying identical PFGE profiles (Figure 1). The five *bla*-$_{CTX-M-15}$ positive and/or TEM-1 positive isolates were nonclonal. Two of ESBL isolates shared genotypes with beta-lactam susceptible isolates indicating dissemination of resistance by horizontal plasmid transfer rather than by clonal spread of the resistant isolates.

Discussion

In this study, five β-lactam resistant *S. sonnei* isolates and six beta-lactam sensitive *S. sonnei*, were subjected to molecular characterization. Amplification of a *bla*-$_{CTX-M}$ gene but not of *bla*-$_{TEM}$ from both total and plasmid DNA preparations from the five beta-lactam-resistant *S. sonnei* isolates indicates that the *bla*-$_{CTX-M}$ encoding gene is plasmid-borne while the *bla*-$_{TEM}$ gene is chromosomal. This observation is concordant with the results of previous studies [2,17,18], which demonstrate that ESBLs found in gram-negative organisms are usually encoded on transferable plasmids.

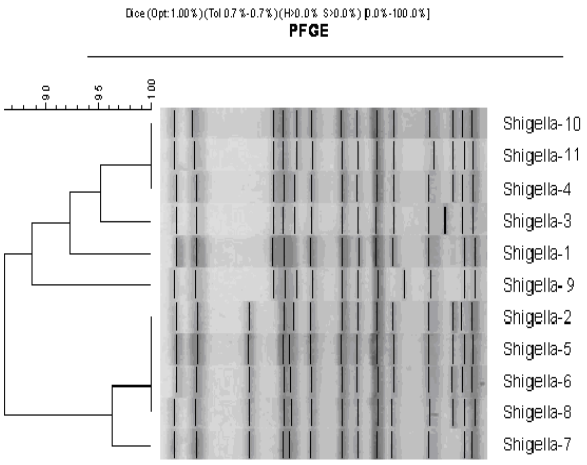
Nucleotide sequence analysis performed on ESBL-producing *S. sonnei* isolates revealed that the plasmid encoded *bla*-$_{CTX-M}$ gene was *bla*-$_{CTX-M-15}$ and that the chromosomally encoded *bla*-$_{TEM}$ gene was *bla*-$_{TEM-1}$.

Conjugation experiments further demonstrated that the *bla*-$_{CTXM-15}$ gene is encoded on an approximately 70-kb plasmid. Cheung *et al*. demonstrated that the *bla*-$_{CTX-M-15}$ identified in *S. sonnei* isolates in Hong Kong was located on a 60-kb plasmid and was associated with the insertion sequence *ISEcp1* located upstream [8]. Andreas *et al.* also showed that the *bla*-$_{CTX-M-2}$ and *bla*-$_{TOHO-1a}$. ESBLs recovered from *S. flexneri* isolates in Argentina were located on mobile genetic elements, which share the same resistance determinants [6]. Kim *et al.* showed that the CTX-M-14-type ESBLs in *S. sonnei* strains isolated in Korea were transmitted through interspecies spread by horizontal transfer of endemic resistance plasmids between medical facilities and the community [1]. The presence of *bla*-$_{CTX-M-15}$ in other *Enterobacteriaceae* in Lebanon [14] suggests that the dissemination of extended-spectrum beta-lactam resistance is mainly due to horizontal transfer of resistance by this plasmid-borne gene.

Table 3: Expression levels of the CTX-M-15 enzyme as compared to the housekeeping GlgC enzyme with and without exposure to Ceftazidime (CAZ) at sub-inhibitory concentrations. Cp= crossing point on the LightCycler™ 2.0 instrument.

	***Shigella sonnei* #1**	***Shigella sonnei* #3**	***Shigella sonnei* #4**
Mean Cp values of *bla*-$_{CTX-M-15}$ + CAZ	21.37	17.77	20.44
Mean Cp values of *bla*-$_{CTX-M-15}$ - CAZ	24.03	21.99	22.12
Mean Cp values of GlgC + CAZ	26.87	22.65	25.79
Mean Cp values of GlgC - CAZ	26.87	23.74	25.79
***bla*-$_{CTX-M-15}$ / GlgC ratio + CAZ**	65.1	52.15	61.66
***bla*-$_{CTX-M-15}$ / GlgC ratio - CAZ**	14.3	8.48	23.65
Relative expression ratio +/- CAZ	4.5 folds	6.1 folds	2.6 folds

Figure 1: Pulsed Field Gel Electrophoresis (PFGE) of the macro-restricted chromosomal DNA on all 11 *Shigella sonnei* isolates.



Macro-restriction analysis by PFGE showed that two ESBL-positive *S. sonnei* isolates had profiles identical to some β-lactam susceptible isolates. This indicates that horizontal inter- and/or within species dissemination of the 70-kb plasmid encoding the *bla*-$_{CTX-M-15}$ gene plays a significant role in the spread of resistance to third-generation cephalosporins. Overall, there are very few band differences between the isolates on PFGE, suggesting that the *S. sonnei* isolates analyzed in this study have limited genetic diversity.

Real time RT-PCR relative quantification performed on ESBL-producing *S. sonnei* isolates demonstrated that transcription of the *bla*-$_{CTX-M-15}$ mRNA is constitutive and independent of exposure to ceftazidime. However, exposure to sub-inhibitory concentrations of ceftazidime increased the level of expression of *bla*-$_{CTXM-15}$ by approximately two- to six-fold as compared to the housekeeping gene *glgC* mRNA. To our knowledge, the level of expression of ESBLs has not been previously tested by this approach.

In conclusion, our study demonstrated the recovery of two additional *S. sonnei* isolates at a tertiary-care center in Lebanon, one ESBL-producing and one non-ESBL β-lacatamase positive isolate. This study, along with the previous investigation, [15] provided a more detailed molecular characterization of the species. The antimicrobial resistance to third-generation cephalosporins was found to be mediated in four of five isolates by the plasmid-borne *bla*-$_{CTXM-15}$ gene and not by clonal dissemination, while in one of five beta-lactamase positive isolates it was mediated by the chromosomally encoded *bla*-$_{TEM-1}$ gene only. Previously documented occurrences of the *bla*-$_{CTX-M-15}$ gene in ESBL-positive *E. coli*, *K. pneumoniae* and other *Enterobacteriaceae* species in Lebanon suggest a spread of the *bla*-$_{CTX-M-15}$ gene among these ESBL-producing *Enterobacteriaceae* isolates [19]. The observed findings may predict future dissemination of resistance to the third-generation cephalosporins among *Shigella* isolates in the Lebanese community, causing serious public health concerns and the need for therapeutic alternatives. For that reason, continued surveillance is needed to assess the overall incidence of ESBL-producing *Shigella* isolates as well as their encoding gene(s) from patients with shigellosis in Lebanon.

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Conflict of interest: No conflict of interest is declared.