Myanmar Health Sciences Research Journal, Vol. 29, No. 3, 2017

Characterization of Cephalosporins Resistant *Shigella sonnei* Isolates in Japan

Myint Thazin Aung[1], Hidemasa Izumiya[2], Eiji Arakawa[2], Masatomo Morita[2] & Makoto Ohnishi[2]*

[1]Department of Laboratory Medicine
International University of Health and Welfare, Japan
[2]National Institute of Infectious Diseases, Japan

Drug resistance in bacteria is a global challenge for 21st century threatening our health security. In this study, we identified and characterized cephalosporin resistant strains of *Shigella sonnei* in Japan, demonstrating that bla_{CTX-M} genotypes were dominant among extended spectrum beta-lactamases (ESBLs) producing strains; $bla_{CTX-M-14}$ (strain D), $bla_{CTX-M-15}$ (strains B and C), and chimera $bla_{CTX-M-64}$ variant (strain C) were detected. After the characterization of upstream and downstream of the bla_{CTX-M} genes in three genotype strains, the order of genetic elements was estimated as follows; ISEcp1, $bla_{CTX-M-14}$ and IS903 for the strain D, ISEcp1, $bla_{CTX-M-15}$ and orf_{477} for the strains B and C, and ISEcp1, $bla_{CTX-M-64}$ and orf_{477} for the strain C, indicating that the genetic order flanking bla_{CTX-M} genes isolated is very similar or identical to the previously reported strains.

Key words: Cephalosporin resistance, *Shigella sonnei*, ESBL, Inverted repeat

INTRODUCTION

Shigella episodes occur throughout the world annually, the majority of which occur in developing countries. The number of patients is estimated as 164.7 million people per year with 1.1 million deaths, 60% of whom are children under the age of 5 and this can result in reduced growth even if they survive.[1] Even in an industrialized country, such as the United States, *Shigella* causes an estimated 500,000 cases of diarrhea every year.[2] Of the four species within the genus, *Shigella flexneri* and *Shigella sonnei* are predominant and now the latter replaces the *Shigella flexneri* as the major species in industrialized regions.[3] *Shigella* spreads easily and rapidly from person to person and through contaminated food and recreational water. Over the last 50 years, *Shigella* has demonstrated extraordinary prowess in acquiring plasmid-encoded resistance to the antimicrobial drugs that previously constituted the first-line therapy. In the 1990s, few reliable options existed to treat multi-resistant *Shigella* infections, particularly in developing countries where cost practicalities are of paramount considerations. Nowadays, emergence of extended-spectrum beta-lactamases (ESBLs) resistance in *Shigella sonnei* is progressively increasing worldwide.[1, 4-6]

In Japan, many *Shigella sonnei* isolates were derived from imported cases[7] and found to have multidrug resistant (MDR) phenotypes to nalidixic acid, tetracycline, and trimethoprim-sulfamethoxazole.[8] An increasing number of *Shigella* isolates are showing resistance to nalidixic acid and other quinolones leading to a therapeutic problem. Patients with HIV infection may develop persistent or recurrent intestinal *Shigella* infections, even in the presence of adequate antimicrobial therapy. They also face an increased risk of *Shigella*

*To whom correspondence should be addressed.
Tel: +81-287-24-3000
E-mail: myintthazinaung@gmail.com

bacteremia, which can be recurrent, severe or even fatal.[9] Emergence of antibiotics resistance in bacteria worldwide and reduction of new antibiotics will threaten our future health.

Among them, the transferable resistance to beta-lactam antimicrobial drugs mediated by production of ESBLs is of particular concern[2] ESBLs are mainly encoded by three important plasmid-mediated enzymes, namely *bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{CTX-M}$ types. CTX-M type extended-spectrum beta-lactamases have recently been the most widespread ESBL in *Enterobacteriaceae*, and replacing classical TEM and SHV ESBLs. The global spread of CTX-M-producing ESBL microorganisms with epidemic resistance plasmids is also associated with multidrug resistance (MDR) producer and virulent high-risk clones.[10]

Most of the MDR bacteria get the drug resistance genes by horizontal gene transfer mechanism amongst different bacteria species and strains.[11] WHO recommends ciprofloxacin as the first-line therapy, with ceftriaxone and pivmecillinam as secondary alternatives for the treatment of shigellosis. However, the situation recently is further worsened by the emergence of ESBL-producing strains worldwide.[12] The purpose of the present study was to determine and characterize the cephalosporin-resistant genes in ESBL-producing *Shigella sonnei* strains isolated in Japan.

MATERIALS AND METHODS

Strains used

In this study, six *Shigella sonnei* strains (strain A, B in 2009, strain C, D in 2010, and strain E, F in 2012) were used for the experiments. The strains were isolated from patients with diarrhoea in the local Public Health Institutes in Japan and sent to Department of Bacteriology I in National Institutes of Infectious Disease (NIID). Three patients had the history of travel before the onset of illness, two were to China (strain C and D) and one was to Turkey (strain F) and the other three patients had no history of foreign travel.

Antimicrobial susceptibility testing

Antimicrobial susceptibility test of 18 anti-biotics (BD BBL™ Sensi-Disc™, Becton Dickinson, USA, Benex Limited) was performed by Modified Kirby Bauer's method.[13] The results were interpreted according to the criteria of the Clinical and Laboratory Standard Institute (CLSI, 2013).[14] Phenotype detection of ESBL-producing strains was done by Epsilometer test (E test, SYSMEX bioMerieux, Co., Ltd.) by using the E test strips containing cefotaxime (CT) and cefotaxime plus clavulanic acid (CTL) (SYSMEX, ESBL CT/CTL) and also ceftazidime (TZ) and ceftazidime plus clavulanic acid (TZL) (SYSMEX, ESBL TZ/TZL).

DNA extraction

Four ESBL-producing isolates of *Shigella sonnei* were subcultured on TSA (Trypticase Soy Agar) media and incubated overnight at 37ºC. For the DNA extraction, a loopful of colony grown on the agar was suspended in a tube containing 100 µl of sterile deionized water, and boiled at 100ºC for 10 minutes and immediately cooled on ice. After centrifugation of the tube at 12,000 g for 5 minutes, the DNA containing supernatant was transferred into a new tube and kept at -30ºC freezer for PCR testing.

Molecular detection of ESBL genes by PCR amplification

DNAs of all the four ESBL-producing isolates were subjected to screening ESBL genotypes by PCR with specific primer pairs of *bla*$_{TEM-1}$, *bla*$_{OXA-1}$ and *bla*$_{CTX-M}$ genes. Primers showed in Table 1 were constructed based on previously published papers and from the sequences of NCBI databases (AB976601.1, AB976604.1, AB9 76605.1, KT201629, and KC576517.1 in Table 1). PCR reactions were optimized using 0.2 µM each of primers, DNA template, PCR buffer 10 µl, dNTP 200 µM,

and Taq polymerase in a total volume of 50 µl. Cycling conditions were as follows; initial denaturation at 94ºC for 3 minutes, 35 cycles of amplification (94ºC for 30 seconds, 55ºC for 45 seconds and 72ºC for 1 minute) followed by 72ºC for 5 minutes with a cold stop at 4ºC using gradient thermal cycler. Amplified PCR products were electrophoresed in 2% agarose gel containing 0.5 µg/ml ethidium bromide in 1X Tris borate EDTA (TBE) buffer. A 100 bp ladder was used as a marker. Finally, the products were visualized by ultraviolet light transillumination.

In order to characterize more specific CTX-M genotypes in the ESBL-producing strains, the specific primer pairs for $bla_{\text{CTX-M-15}}$ and $bla_{\text{CTX-M-14}}$ genes were constructed from the sequence of NCBI database (Table 1). The conserved upstream region ISEcp1 for the CTX-M-1 and CTX-M-9 clusters was used for construction of additional primer pairs for both genes. For the downstream region, orf_{477} was usually found in CTX-M-1 cluster whereas IS903 was more common than orf_{477} in the CTX-M-9 cluster. For a chimeric strain with $bla_{\text{CTX-M-64}}$ gene (CTX-M-1 cluster), the variety of primer pairs were constructed from upstream ISE-cp-1 region, CTX-M-15 homology sites, CTX-M-14 homology region and down-stream orf_{477} region by using the NCBI database (Table 1). PCR cycling conditions were slightly modified as follows; initial denaturation at 94ºC for 5 minutes, 35 cycles of amplification (94ºC for 30 seconds, 58ºC for 45 seconds and 72ºC for 2 minutes) followed by 72ºC for 5 minutes with a cold stop at 4ºC using gradient thermal cycler.

DNA sequencing for the determination of CTX-M genotypes and surrounding regions

For DNA sequences, the amplified PCR products were purified by using Qiagen purification kit (Qiagen, Valencia, California, USA) and then DNA sequencing was done for both strands of amplified DNA fragments from all strains. Each sequence reaction mixture consisted of 40 ng/µl purified amplicon, 1.6 µl of 1pmole primer (CTX-M Fw, CTX-M Rv, TEM-1 Fw, TEM-1 Rv, ISEcp1-C Fw, orf_{477} Rv1 and orf_{477} Rv2) and 4 µl of ABI BigDye terminator V3.1 cycle sequencing kit (Applied Biosystems, USA).

The sequence reaction was done as follows; initial denaturation at 95ºC for 1 minute followed by 30 cycles consisting of denaturation at 95ºC for 10 seconds, annealing at 50ºC for 20 seconds, and elongation at 60ºC for 4 minutes. The PCR products were purified with spin column method and 20 µl of purified product was sequenced in ABI 3730 genetic analyzer. The resulting DNA sequences were analyzed with the Genbank sequence databases by using the NCBI BLAST program.

RESULTS

In this study, 6 strains of *Shigella sonnei* were subjected to antimicrobial susceptibility testing, and 4 strains among them showed resistance to cefotaxime (Table 2). According to the antimicrobial susceptibility testing, all the four cefotaxime resistant strains were resistant to ampicillin, streptomycin and cephalothin but susceptible to imipenem, meropenem, ciprofloxacin, amikacin, fosfomycin, cefotetan, cefoxitin and chloramphenicol.

A majority of the isolates were resistant to septrin, tetracycline, gentamicin, kanamycin, sulfisoxazole, and nalidixic acid. All *Shigella* isolates tested had high susceptibility (100%) to imipenem, meropenem, ciprofloxacin, amikacin, fosfomycin, cefotetan, cefoxitin and chloramphenicol. As the four cefotaxime resistant strains (strains B, C, D and E) were sensitive to cephamycins (cefotetan, and cefoxitin) and carbapenems (imipenem, meropenem), they were suspected to produce ESBLs. To determine whether the susceptibility of cefotaxime-resistant strains is reduced by the inhibitor of ESBLs

Table 1. Oligonucleotide primers used in this study

Name of primers (Amplified length) (bp)		Target reference	Sequences (5'-3')	Position
Primer pairs for bla genes				
(i) CTX-MFW	544	*bla* $_{CTX-M}$[15]	TTTGCGATGTGCAGTACCAGTAA	205-227
CTX-M Rv			CGATATCGTTGGTGGTGCCATA	748-727
(ii) TEM Fw	861	*bla*$_{TEM-1}$[16]	ATGAGTATTCAACATTTCCGTG	1-22
TEM Rv			TTACCAATGCTTAATCAGTGAG	861-840
(iii) OXA Fw	797	bla$_{OXA-1}$[17]	TTGTTAGCCGTTAAAATTAA	22-40
OXA Rv			GTTAAATTCGACCCCAAGTT	818-799
Primer pairs for bla $_{CTX-M-14}$ gene, and its upstream and downstream				
(iv) CTX-M-14 Fw1	876	N-moiety of bla$_{CTX-M-14}$	ATGGTGACAAAGAGAGTGCAA	147-167
CTX-M-14 Rv1		C-moiety of bla$_{CTX-M-14}$ AB976605.1	T TACAGCCCTTCGGCGATGAT	1022-1002
(v) ISEcp1-C FW	1104	ISEcp1 for CTXM1/9 clusters	GTGTTGCTCTGTGGATAACT	1019-1038
CTX-M-14 Rv1		C-moiety of bla$_{CTX-M-14}$ AB976601.1	TTACAGCCCTTCGGCGATGAT	1022-1002
(vi) CTX-M-14N Fw2	1030	N-moiety of bla$_{CTX-M-14}$	GATGGTGACAAAGAGAGTGC	140-159
CTX-M-14IS Rv2		IS903 AB976604.1	GTTATGGAGCCACGGTTGAT	1169-1150
Primer pairs for bla $_{CTX-M-15}$ gene				
(vii) CTX-M-15 Fw	866	N-moiety of blaCTX-M-15	AATCACTGCGCCAGTTCACG	137-156
CTX-M-15 Rv		C-moiety of blaCTX-M-15 KT201629	TTACAAACCGTCGGTGACGA	1002-983
(viii) ISEcp1 Fw	934	ISEcp1	AACACACGTGGAATTTAGG	61-79
CTX-M-15C Rv		C-moiety of bla$_{CTX-M-15}$ KT201629	CGTCGGTGACGATTTTAGCC	994-975
(ix) ISEcp1-C Fw	1126	ISEcp1 for CTXM1/9 clusters	GTGTTGCTCTGTGGATAACT	1019-1038
ORF477 Rv		orf$_{477}$ for bla$_{CTX-M-15}$ AB976601.1	TTTCCCCATTCCGTTTCCGC	2144-2125
Primer pairs (Fw and Rv) specific for bla$_{CTX-M-64}$ genes				
(x) ISEcp1-C Fw	723	ISEcp1 for CTXM1/9 clusters	GTGTTGCTCTGTGGATAACT	1019-1038
CTX-M64-14 Rv		*bla*$_{CTX-M14}$ homology site of *bla*$_{CTX-M-64}$ AB976601.1	CAGACGAAACGTCTCATCGC	1741-1722
(xi) CTX-M64-14 Fw	545	*bla*$_{CTX-M-14}$ homology site of *bla*$_{CTX-M-64}$	ATGGCGCAGACGTTGCGTCA	1811-1830
ORF477 RV1		orf$_{477}$ for *bla*$_{CTX-M-64}$ AB976601.1	AACCTGGCATGCCGTGCTGT	2355-2336
(xii) CTX-M-64-15 Fw	347	*bla*$_{CTX-M-15}$ homology site of *bla*$_{CTX-M-64}$	AAAGATCGTGCGCCGCTGAT	2009-2028
ORF477 Rv1		orf$_{477}$ for *bla*$_{CTX-M-64}$ AB976601.1	AACCTGGCATGCCGTGCTGT	2355-2336
(xiii) CTX-M-64-15 Fw	800	*bla*$_{CTX-M-15}$ homology site of *bla*$_{CTX-M-64}$	AAAGATCGTGCGCCGCTGAT	1001-1020
ORF477 Rv2		orf$_{477}$ for *bla*$_{CTX-M-64}$ KC576517.1	GGTGAGATTTTTGGCGTAGG	800-1781

(clavulanic acid; CVA) Epsilometer test (E test) was used in this study. All four cefotaxime-resistant strains showed 8 fold reduction of the MIC for cefotaxime or ceftazidime (all three strains except for strain D) in combination with clavulanic acid β lactamase inhibitor) than the MIC without inhibitor, which indicated they can be considered as ESBL producers according to CLSI 2013 guidelines (Table 3). The presence of gene conferring the ESBL phenotypes was examined by PCR with *bla*$_{CTX-M}$, *bla*$_{TEM-1}$, and *bla*$_{OXA-1}$ specific consensus primers [Table 1(i), (ii) and (iii)]. Regarding the molecular detection of ESBL encoding genes, all four cefotaxime-resistant isolates were positive for the primers of *bla*$_{CTX-M}$ but not for *bla*$_{TEM-1}$ and *bla*$_{OXA-1}$, while only strain B showed positive for the *bla*$_{TEM-1}$ primers. PCR-amplified DNA fragments with the *bla*$_{CTX-M}$ primers were sequenced for both strands by using ABI 3130 genetic analyzer and the sequences encountered were *bla*$_{CTX-M-15}$ in strains B and E, *bla*$_{CTX-M-14}$ in strain D, and chimera *bla*$_{CTX-M-64}$ in the strain C with 100% identity of sequences in NCBI databases by aligning the database of NCBI BLAST program. Upstream and downstream of each *bla*$_{CTX-M}$ gene are reported to be diverse; Insertion sequences (ISs) have been identified in the

Table 2. Antibiotics sensitivity result

Drugs	AMP	TC	CP	SM	ST	G25	FOM	KAN	GEN	AMK	NAL	CIP	CET	CIT	CFX	CTX	IPM	MEPM
Disk constant	10 µg	30 µg	30 µg	10 µg	23.75/ 1.25 µg	0.25 mg	200 µg	30 µg	10 µg	30 µg	30 µg	5 µg	30 µg	30 µg	30 µg	30\ µg	10 µg	10 µg
R≤	13	11	12	11	10	12	12	13	12	14	13	15	14	12	14	22	19	19
I (range)	14-16	12-14	13-17	12-14	11-15	13-16	13-15	14-17	13-14	15-16	14-18	16-20	15-17	13-15	15-17	23-25	20-22	20-22
S≥	17	15	18	15	16	17	16	18	15	17	19	21	18	16	18	26	25	23
Strain A	S	S	S	R	S	S	S	S	S	S	S	S	R	S	S	S	S	S
Strain B	R	S	S	R	S	S	S	S	S	S	S	S	R	S	S	R	S	S
Strain C	R	R	S	R	R	R	S	I	R	S	R	S	R	S	S	R	S	S
Strain D	R	R	S	R	R	R	S	R	R	S	R	S	R	S	S	R	S	S
Strain E	R	S	S	R	S	S	S	S	S	S	S	S	R	S	S	R	S	S
Strain F	S	S	S	R	S	S	S	S	S	S	S	S	I	S	S	S	S	S

AMP=Ampicillin, TC=Tetracycline, CP=Chloramphenicol, SM=Streptomycin, SXT=Septrin, CTT=Cefotetan, G25=Sulfisoxazole, FOM=Fosfomycin, KAN=Kanamycin, GEN=Gentamicin, AMK=Amikacin, CFX=Cefoxitin, NAL= Nalidixic acid, CIP= Ciprofloxacin, CET=Cephalothin, CTX=Cefotaxime, MEPM=Meropenem, IPM=imipenem, S=Susceptible, R=Resistance, I=Iintermediate

Table 3. MIC levels (µg/ml) of six strains with and without clavulanic acid by Epsilometer test

E test	CAZ	CAZ+CVA	CTX	CTX+CVA	Presence of ESBL(+/-)
Strain A	‹0.5	0.94	‹0.25	‹0.25	(-)
Strain B	6	0.94	›16	0.16	(+)
Strain C	8	0.19	›16	0.23	(+)
Strain D	‹0.5	0.64	›16	0.16	(+)
Strain E	›32	0.94	›16	0.16	(+)
Strain F	‹0.5	0.64	‹0.25	‹0.16	(-)

CAZ=Ceftazidine, CTX=Cefotaxime, CVA=Clavulanic acid

upstream of the *bla*$_{\text{CTX-M}}$ genes, including ISEcp1, ISCR1, IS10 and IS26. ISEcp1 with variable distances from each *bla*$_{\text{CTX-M}}$ gene is widely found in the upstream of different *bla*$_{\text{CTX-M}}$ genes which are associated with all CTX-M clusters except for the CTX-M-8 cluster.[10, 18] Upstream regions of *bla*$_{\text{CTX-M-14}}$, *bla*$_{\text{CTX-M-15}}$ and *bla*$_{\text{CTX-M-64}}$ genes have insertion sequence (IS) of ISEcp1[10] and downstream regions of *bla*$_{\text{CTX-M-14}}$, *bla*$_{\text{CTX-M-15}}$ and *bla*$_{\text{CTX-M-64}}$ genes have IS$_{903}$, orf$_{477}$ and orf$_{477}$, respectively.[18] Therefore, the specific primer pair sets (Table 1) were constructed by using the upstream ISEcp1 sequence, the N and C-terminal sequence portions of the specific CTX-M genes, and the downstream orf$_{477}$ and IS$_{903}$ sequences in order to get more specific information on the flanking regions of each *bla*$_{\text{CTX-M}}$ gene.

All four ESBL-producing strains were examined by using the primer targeted for the ISEcp1 region (ISEcp1-C Fw primer in Table 1) and the primers targeted orf$_{477}$ (ORF477 Rv1/Rv2 in Table 1). Strains B, C and E showed positive reaction, indicating that the strains B and E (*bla*$_{\text{CTX-M-15}}$) and also strain C (*bla*$_{\text{CTX-M-64}}$) carried ISEcp1 in their upstream and orf$_{477}$ in their downstream. Strain D was positive for the primer set with ISEcp1-C-Fw and CTX-M-14IS Rv2 (primer targeted for IS903 in Table 1), indicating that strain D carried ISEcp1 in its upstream and IS903 in its downstream.

Further characterization of surrounding region of the strain D was done. The location of ISEcp1 and IS903 against *bla*$_{\text{CTX-M-14}}$ gene was determined by PCR using primer set of ISEcp1-C Fw (ISEcp1 location) and CTX-M-14Rv1 (C-moiety of *bla*$_{\text{CTX-M-14}}$), and primer set of CTX-M-14N Fw2 (N-moiety of *bla*$_{\text{CTX-M-14}}$ gene) and CTX-M-14IS Rv2 (location of IS903), respectively, resulting in the positive reaction with estimated size of amplified fragments. The result showed that the genes are in the order of ISEcp1, *bla*$_{\text{CTX-M-14}}$ and IS903 (Fig. 1). The location of ISEcp1 and orf$_{477}$ against the *bla*$_{\text{CTX-M-15}}$ in strains B and E was also determined by PCR using primer set of ISEcp1Fw (ISEcp1 location) and CTX-M-15C Rv (C-moiety of *bla*$_{\text{CTX-M-15}}$), and primer set of ISEcp1-C-Fw (ISEcp1 location) and ORF477 Rv (location of orf$_{477}$), respectively, resulting in the positive reaction with estimated size of amplified

fragments. The result showed that the genes are in the order of ISEcp1, *bla*$_{CTX-M-15}$ and orf$_{477}$. Figure 1 shows the estimated genetic order around *bla*$_{CTX-M-14/15/64}$ genes in this study and from previous reports.[10, 18, 27]

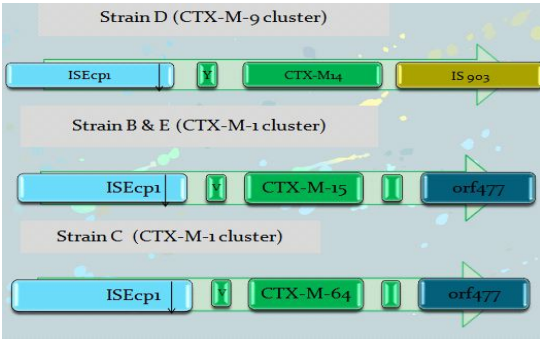

Fig. 1. Structure of ESBL-producing CTX-M gene of all strains

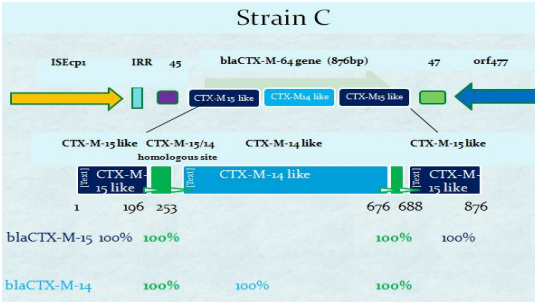

Fig. 2. Structure of chimeric *bla*$_{CTX-M-64}$ gene

The *bla*$_{CTX-M-64}$ gene is known as a chimeric generic element of *bla*$_{CTX-M-14}$ and *bla*$_{CTX-M-15}$ genes; N and C-terminus components of CTX-M-64 are derived from of CTX-M-15 and the middle portion is from CTX-M-14.[19] In order to get more information and to identify chimeric *bla*$_{CTX-M-64}$ gene of strain C, further experiments were done by the use of PCR. Sets of primers used were as follows; ISEcp1-C Fw (target for ISEcp1) and CTX-M64-14 Rv (*bla*$_{CTX-M-14}$ homologous site in the middle of *bla*$_{CTX-M-64}$), CTX-M64-14 Fw (*bla*$_{CTX-M-14}$ homologous site in the middle of *bla*$_{CTX-M-64}$) and ORF477 Rv1 (orf$_{477}$), and CTX-M64-15 Fw (*bla*$_{CTX-M-15}$ homologous site in the end of *bla*$_{CTX-M-64}$) and ORF477 Rv1 or Rv2 (orf$_{477}$). The results by PCR gave positive reaction with estimated size of amplified fragments from the genetic structure previously reported.[19]

The genetic order of *bla*$_{CTX-M-64}$ gene of strain C was estimated as ISEcp1, chimeric of *bla*$_{CTX-M-15}$ and *bla*$_{CTX-M-14}$, and orf$_{477}$ (Fig. 1). Amplified fragments were sequenced, and the results showed that chimeric *bla*$_{CTX-M-64}$ and flanking regions of ISEcp1 and orf$_{477}$ were 100% identical with the previously reported sequences Figure 2 shows demonstrates the structure of a chimeric generic element of *bla*$_{CTX-M-64}$ gene from the sequenced data of each PCR-amplified fragment obtained in this study and from previous reports.[19, 21]

DISCUSSION

Shigellosis is one of the diseases with high mortality under the age of five and its episodes occur worldwide annually.[1] In developed countries, *Shigella sonnei* is the most frequently isolated species of *Shigella*.[3] Nowadays, WHO recommends ciprofloxacin as the first-line drug with ceftriaxone and pivmecillinam as the secondary alternatives, however, the number of quinolones-resistant *Shigella* isolates is increasing in developing countries and also ESBL-producing isolates are emerging.[9, 12] Thus, this study addressed the issue of the presence of cephalosporin and other resistance in *Shigella sonnei* and the molecular analysis of cephalosporin resistance genes.

All the six *Shigella sonnei* strains used were 100% susceptible to imipenem, meropenem, chloramphenicol, ciprofloxacin, amikacin, and fosfomycin. However, results from other countries showed varying rate of resistance to ciprofloxacin; India (82%), Eastern Nepal (28.3%), China (25.2%), and Northern Ethiopia (6.7%).[22-24] Four isolates of six *Shigella sonnei* were resistant to cefotaxime and its resistance was inhibited with addition of clavulanic acid, resulting in the ESBLs. Among ESBL-

producing *Shigella sonnei* isolates, bla_{CTX-M} genes were dominant in worldwide. All of the ESBL-producing isolates in this study carried bla_{CTX-M} genes, being consistent with the other studies;[18, 19] 91.8% bla_{CTX-M} types in *Shigella sonnei* were reported in China.[25]

CTX-M enzymes are more active against cefotaxime and ceftriaxone than ceftazidime, but point mutations can increase activity against ceftazidime; thus CTX-M-15 and -32 from CTX-M-1 and -3 clusters, respectively, differ solely by Asp-240/Gly substitutions, resulting in 100-fold more actively against ceftazidime.[26-29] CTX-M-1 cluster ($bla_{CTX-M-15}$ positive strains B and E, and $bla_{CTX-M-64}$ positive strain C) showed similar findings with high activity against ceftazidime (Table 3), while the MIC of ceftazidime in CTX-M-9 cluster ($bla_{CTX-M-14}$ positive strain D) was found in the susceptibility range (<0.5 μg/ml) whereas ESBL detection is based on cefotaxime utilization in this study (Table 3).

Origin of each CTX-M cluster is believed to be derived from the chromosomal *bla* genes present in different *Kluyvera* species.[10] One hundred and fifty variants of CTX-M genes can be grouped into six clusters according to their genetic relatedness: namely CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9, CTX-M-25 and KLUC. There is less than 90% identity between clusters and more than 94% identity within clusters.[18]

Among them, the CTX-M-1 and CTX-M-9 clusters are the most frequently reported groups worldwide, and $bla_{CTX-M-15}$ and $bla_{CTX-M-14}$ are the most common variants within these groups.[30] Several chimeras of CTX-Ms have been reported since 2009 and the most commonly found chimeras (new mutant genotypes) are $bla_{CTX-M-64}$, $bla_{CTX-M-123}$ and $bla_{CTX-M-132}$ which contain at least three hybrid sites of $bla_{CTX-M-14}$ and $bla_{CTX-M-15}$.[19, 20] Similarly, in this study of *Shigella sonnei* in Japan, of CTX-Ms such as $bla_{CTX-M-15}$, $bla_{CTX-M-14}$, and chimera $bla_{CTX-M-64}$ genes were found.

Surrounding structures of CTX-M beta-lactamase genes have been mainly generated by the incorporation of genetic mobilization units such as insertion sequences (ISEcp1 or ISCR1), complex class 1 integrons and transposons.[18] In the present study, all the upstream and downstream of $bla_{CTX-M-15}$ and chimera $bla_{CTX-M-64}$ genes were flanked by ISEcp1 and orf_{477}, respectively. The upstream and downstream of $bla_{CTX-M-14}$ gene was flanked by ISEcp1 and IS903, respectively. These findings are also consistent with the other studies[10, 18]

In 2010, chimera $bla_{CTX-M-64}$ carrying $bla_{CTX-M-15}$ (N- & C- terminal domains) and $bla_{CTX-M-14}$ (central domain) was found in Japan and in China.[19-21] The chimera $bla_{CTX-M-64}$ strain (strain C) in this study was identical to the previously reported $bla_{CTX-M-64}$ and also flanked by ISEcp1 and orf_{477} in the upstream and downstream, respectively, carrying the 45 base pairs spacer between ISEcp1 and $bla_{CTX-M-64}$ gene and the 47 base pairs spacer between $bla_{CTX-M-64}$ and orf_{477} identical to the previous (Fig. 2).

The current global spread of multidrug-resistant gram-negative bacteria especially ESBL-producing bacteria is increasing. The increase of CTX-M producers has led to an increasing use of carbapenems, resulting in the emergence of carbapenem-resistant *Enterobacteriaceae*.[31] Most of ESBLs in the *Shigella* species are carried by widely transferrable IncFII[32, 33] and IncK[34] plasmids with resistance to other antibiotics, and it could cause the threat of human security.

This study may not be representative of the community prevalence of *Shigella sonnei* because of small sample size; however, the findings showed the characteristic features of the current circulating ESBL-producing *Shigella sonnei* strains. In order to get more detailed information, further study should be continued to monitor ESBL-producing bacteria and explore the mechanisms of spread of CTX-M genotypes.

ACKNOWLEDGMENT

We acknowledge Professor Haruo Watanabe for the critical reading and revising of the manuscript. We also specially thank Professor Takashi Kurata, Professor Yoshihiro Kitamura and Professor Mariko Y Momoi in International University of Health and Welfare (IUHW) to conduct this study in NIID. We would like to thank local Public Health Institutes for providing *Shigella sonnei* strains used in this study.

REFERENCES

1. Kotloff KL, Winikoff JP, Ivanoff B, *et al*. Global burden of *Shigella* infections: Implications for vaccine development & implementation of control strategies. *Bulletin of the World Health Organization* 1999; (77): 651-666.
2. Antibiotic resistance threats in the United States. CDC: Centers for disease control and prevention. [Internet] 2013[update 2013 April 23]. Available from: http://www.cdc.gov› threat-report.
3. Thompson CN, Duy PT & Baker S. The rising dominance of *Shigella sonnei*: An inter-continental shift in the etiology of Bacillary Dysentery. *PLOS Neglected Tropical Diseases* 2015; 6: DOI:10.1371/ journal. pntd.0003708
4. DeLappe N, O'Halloran F, Fanning S, *et al*. Antimicrobial resistance and genetic diversity of *Shigella sonnei* isolates from western Ireland, an area of low incidence of infection. *Journal of Clinical Microbiology* 2003; 41: 1919-1924.
5. Mammina C, Pontello M, Dal Vecchio A & Nastasi A. The *Shigella sonnei* Working Group. Identification of *Shigella sonnei* biotype g isolates carrying class 2 integrons in Italy (2001 to 2003). *Journal of Clinical Microbiology* 2005; 43: 2467-2470.
6. Oh JY, Yu HS, Kim SK, *et al.* Changes in patterns of antimicrobial susceptibility and integron carriage among *Shigella sonnei* isolates from southwestern Korea during epidemic periods. *Journal of Clinical Microbiology* 2003; 41: 421-423.
7. Infectious Agents Surveillance Reports (IASR). Shigellosis, Japan, 2006-20093 0:311-313. *IASR* [Internet] 2009 [update December 2009] Available from: http://www.idsc.nih.go.jp/iasr/ 30/ 358/tpc358.html
8. Hirose K, *et al.* Antimicrobial susceptibility of *Shigella sonnei* isolates in Japan and molecular analysis of *Shigella sonnei* isolates with reduced susceptibility to fluoroquinolones. *Antimicrobial Agents and Chemotherapy* 2005; 49(3): 1203-1205.
9. Kristjansson M, Viner B & Maslow JN. Polymicrobial and recurrent bacteremia with *Shigella* in patient with AIDS. *Scandinavians Journal of Infectious Diseases*, 1994; 26: 411-416.
10. Cantón R, González-Alba JM & Galán JC. CTX-M enzymes: Origin and diffusion. *Frontier. Microbiology* [Internet]. 2012 [updated 2012 April 2] Available from: http://www.dx. doi.org/10.3389/fmicb.2012.00110
11. Hawkey PM & Jones AM. The changing epidemiology of resistance. *Journal of Antimicrobial Chemotherapy* 2009; 64(Suppl 1): i3-10.
12. WHO. The treatment of diarrhoeal: A manual for physicians and other senior health workers. Geneva. [Internet] 2005[update 2005 April 23]. Available from: http://www.Whqlibdoc. who.int/ publications/ 2005/9241593180.pdf.
13. Bauer AW, Kirby WM, Sherris JC & Turck M. Antibiotic susceptibility testing by a standardized single disk method. *American Journal of Clinical Pathology* 1966; 36: 493-496.
14. Clinical and Laboratory Standards Institute (CLSI). Performance standards for antimicrobial susceptibility testing; 2013: 23rd informational supplement. CLSI document M100-S23. Clinical and Laboratory Standards Institute, Wayne, PA.
15. Edelstein M, *et al*. Prevalence of molecular epidemiology of CTX-M ESBL producing *Escherichia coli* & *Klebsiella pneumoniae* in Russian hospitals. *Antimicrobial Agents and Chemotherapy* 2003; 47: 3724-3732.
16. Sanguinetti M, *et al*. Characterization of clinical isolates of *Enterobacteriaceae* from Italy by the BD Phoenix ESBL detection method. *Journal of Clinical Microbiology* 2003; 41: 1463-1468.
17. Siu LK, *et al*. Beta lactamase in *Shigella flexneri* isolate from Hong Kong, Shanghai & a novel OXA-1 like β-lactamase OXA-30. *Antimicrobial Agents and Chemotherapy* 2000; 44: 2034-2038.
18. Bonnet R. Growing group of extended spectrum beta-lactamases: The CTX-M enzymes. *Antimicrobial Agents and Chemotherapy* 2004; 48: 1-14.
19. Nagano Y, Nagano N, Wachino J, Ishikawa K. & Arakawa Y. Novel chimeric beta-lactamase CTX-M-64, a hybrid of CTX-M-15-like and CTX-M-14 beta-lactamases, found in a *Shigella sonnei* strain resistant to various oxyimino-cephalosporins, including ceftazidime. *Antimicrobial Agents and Chemotherapy* 2009; 53(1): 69-74.

20. Boyd DA, Tyler S, Christiansan S, *et al*. Complete nucleotide sequence of a 92 kilobase plasmid harboring the CTX-M-15 extended spectrum beta-lactamases involved in an outbreak in long-term care facilities in Toronto, Canada. *Antimicrobial Agents and Chemotherapy* 2004; 48(10): 3758-3764.

21. Sun Y, Zeng Z, Chen S, *et al*. High prevalence of blaCTX-M extended-spectrum β-lactamase genes in *Escherichia coli* isolates from pets and emergence of CTX-M-64 in China. *Clinical Microbiology and Infection* 2010; 16(9): 1475-1481.

22. Bhattacharya D, Sugunan AP, Bhattacharya B, *et al*. Antimicrobial resistance in *Shigella*-rapid increase and widening of spectrum in Andaman islands, India. *Indian Journal of Medical Research* 2012; 135: 3654-3670.

23. Bhattacharya S, Khanal B, Bhattarai NR & Das ML. Prevalence of *Shigella* species and their antimicrobial resistance patterns in Eastern Nepal. *Journal of Health Population and Nutrition* 2005; 23(4): 339-342.

24. Gebrekidan A, *et al*. Prevalence and anti-microbial susceptibility patterns of *Shigella* among acute diarrhoeal outpatients in Mekelle hospital, Northern Eithiopia. *BioMed Central Research Notes* 2015; 8: 6-11.

25. Sangeetha AV, *et al*. Clinical and microbiolo-gical profiles of *shigellosis* in children. *Journal of Health Population and Nutrition* 2014; 32(4): 580-586.

26. Ripoll A, Bauero F, Novais, A, *et al*. *In vitro* selection of variants resistant to β-lactams plus β-lactamase inhibitors in CTX- M β-lactamase: Predicting the *In vivo* scenario? *Antimicrobial Agents and Chemotherapy* 2011; 55: 4530-4536.

27. Jacoby GA. Genetics of extended-spectrum of β-lactamases. *European Journal of Clinical Microbiology and Infectious Diseases* 1994; 13: 2-11.

28. Poirel L, Gniadkowski M & Nordmann P. Biochemical analysis of the ceftazidime-hydrolysing extended-spectrum β-lactamase CTX-M-15 and of its structurally related β-lactamase CTX-M-3. *Journal of Antimicrobial Chemotherapy* 2002; 50: 1031-1034.

29. Cartelle M, del Mar TM, Molina F, *et al.* High-level resistance to ceftazidime conferred by a novel enzyme, CTX-M-32, derived from CTX-M-1 through a single Asp240-Gly substitution. *Antimicrobial Agents Chemotherapy* 2004; 48: 2308-2313.

30. Zong Z, Partridge SR, Thomas L & Iredell JR. Dominance of bla_{CTX-M} within an Australian extended-spectrum β-lactamase gene pool. *Antimicrobial Agents Chemotherapy* 2008; 52: 4198-4202.

31. Chong Y, Ito Y & Kamimura T. Genetic evolution and clinical impact in extended spectrum β-lactamase producing *Escherichia coli* and *Klebsiella pneumoniae*. *Infection Genetics and Evolution* 2011; 11: 1499-1504.

32. Coque TM, Novais A, Carattoli A, *et al.* Dissemination of clonally related *Escherichia coli* strains expressing extended spectrum beta-lactamase CTX-M-15. *Emerging Infectious Diseases* 2008; 14: 195-200.

33. Kim JS, Kim J, Jeon SE, *et al.* Complete nucleotide sequence of the IncI1 plasmid pSH4469 encoding CTX-M-15 extended-spectrum beta-lactamase in a clinical isolate of *Shigella sonnei* from an outbreak in the Republic of Korea. *International Journal of Antimicrobial Agents* 2014; 44(6): 533-537.

34. Cottell JL, Webber MA, Coldham NG, *et al.* Complete sequence and molecular epidemiology of IncK epidemic plasmid encoding $bla_{CTX-M-14.}$ *Emerging Infectious Diseases* 2011; 17: 645-652.