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Research article

Molecular characterization of extended-spectrum cephalosporin and fluoroquinolone resistance genes in *Salmonella* and *Shigella* isolated from clinical specimens in Thailand

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ABSTRACT

Antimicrobial resistance of *Salmonella* and *Shigella* has become a major clinical and public health problem. The incident of co-resistance to third generation cephalosporins and fluoroquinolone is a serious therapeutic issue in Thailand. The present study aimed to investigate the antimicrobial resistance and molecular character of clinical *Shigella* and *Salmonella* isolates. A total of 33 *Salmonella* and 53 *Shigella* cefotaxime-resistant isolates were collected from human clinical cases in Thailand during the period from 2011–2018. The antimicrobial susceptibility of *Salmonella* and *Shigella* was determined by the disk diffusion method, and extended-spectrum beta-lactamase (ESBL) production was characterized by the double-disk synergy test. Genotype characterization was performed by PCR and DNA sequencing. Thirty-two (97.0%) and fifty-two (98.1%) isolates of cefotaxime-resistant *Salmonella* and *Shigella*, respectively, were identified as ESBL producers. *Shigella sonnei* (4 isolates), *Salmonella* serovar 4,5,12:i:- (6 isolates), *Salmonella* serovar Agona (2 isolates) and *Salmonella* serovar Rissen (2 isolates) showed co-resistance to ciprofloxacin and cefotaxime or ceftriaxone. The combination of $bla_{\text{CTX-M-15}}$ plus other ESBL and/or AmpC β-lactamase genes was the most dominant of the genotype patterns in ESBL-producing isolates. The plasmid harbouring the *aac(6′)-Ib-cr* gene and mutations of *gyrA* (S83F, D87Y or D87G) and *parC* (T57S) genes was found in 2 ESBL-producing *Salmonella* isolates. Three *Shigella sonnei* isolates harboured mutations in *gyrA* (S83L, D87Y or D87G), and only one *Shigella sonnei* phase I isolate showed mutations in both *gyrA* (S83L and D87G) and *parC* (S80I) genes. Among these clinical *Shigella sonnei* isolates, *qnrS* determinants were identified. Production of ESBLs is an important mechanism for resistance to extended-spectrum cephalosporins in *Salmonella* and *Shigella*. The emergence of a decreased susceptibility to extended-spectrum cephalosporins and fluoroquinolone in ESBL-producing isolates has important clinical and therapeutic implications.

1. Introduction

Non-typhoidal *Salmonella* and *Shigella* strains cause major diarrheal diseases in developing countries (Majowicz et al., 2010; Niyogi, 2005). High levels of multidrug resistant (MDR) non-typhoidal *Salmonella* and *Shigella* strains isolated from clinical specimens resistant to third generation cephalosporins have been detected in Asia (Ji et al., 2010; Lee et al., 2009). The production of extended-spectrum-lactamases (ESBLs) is a major resistance mechanism to extended-spectrum cephalosporins (ESCs) in *Salmonella* and *Shigella* (Pulsrikarn et al., 2017; Ji et al., 2010; Kulwichit et al., 2007). Although ESBL-producing strains of *Salmonella* and *Shigella* have been relatively rare in comparison to other enteric pathogens over the last few decades, an increasing number of these organisms are resistant to at least three different antibiotic classes (Yah, 2010).

According to a previous study, most ESBL genes are encoded by plasmids, some of which also carry genes providing resistance to other antimicrobials such as fluoroquinolones, trimethoprim-sulfamethoxazole, tetracyclines, and aminoglycosides (Woodford et al., 2009). The plasmid-mediated quinolone resistance (PMQR) genes have frequently been associated with the ESBL phenotypes conferred by the bla_{TEM}, bla_{OXA} and $bla_{\text{CTX-M}}$ genes (Doumith et al., 2012). The mechanisms of high-level

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fluoroquinolone resistance are chromosomal mutations in the quinolone resistance-determining regions (QRDR) with changes in either DNA gyrase (*gyrA* and *gyrB*) and/or topoisomerase IV (*parC* and *parE*) genes (Giraud et al., 2006). However, fluoroquinolone resistance isolates carried PMQR determinants associated with low-level fluoroquinolone resistance. The genes encoding PMQR are *qnr* genes (*qnrA*, *qnrB*, *qnrC*, *qnrS* and *qnrV*), efflux pump genes (*qepA* and *oqxAB*) and a variant of aminoglycoside acetyl transferase (*aac-(6′)-lb-cr*) (Jacoby et al., 2014; Robicsek et al., 2006a,b,c). Resistance to quinolones can be mediated by plasmids that produce the quinolone resistance protein (Qnr). Bacterial DNA was protected from fluoroquinolone lethal inhibition by Qnr protein through competitive bind to DNA gyrase and topoisomerase IV. The other gene is *aac-(6′)-lb-cr* gene is the variation of *aac-(6′)-lb* gene also known as genes encoding for aminoglycoside acethyltransferase, responsible for resistance to tobramycin, amikacin, and kanamycin (Robicsek et al., 2006a,b,c). The co-existence and dissemination of plasmids containing ESBL and PMQR genes by ESBL-producing Enterobacteriaceae is a major public health concern (Rodríguez-Martínez et al., 2011).

Previous research demonstrated the high prevalence of ESBL-producing and MDR Enterobacteriaceae in Thai patients (Kiratisin et al., 2008). Considering previous data from Thailand, the possibility of multidrug resistant *Salmonella* and *Shigella* is a public health problem (Pulsrikarn et al., 2017; Sirichote et al., 2010; Hiranrattana et al., 2005). The rising trend of third-generation cephalosporins and ciprofloxacin resistance in clinical *Salmonella and Shigella* isolates is concerning because these drugs are commonly used to treat community-acquired invasive bacterial infections. However, few studies have published genotype data on antimicrobial resistance to ESCs and fluoroquinolone in clinical *Salmonella* and *Shigella* isolates (Chung et al., 2015; Archambault et al., 2006). The present study aimed to investigate the antimicrobial susceptibility to ESCs and fluoroquinolone, as well as the characterization of resistance genes (ESBL, QRDR and PMQR genes) in cefotaxime-resistant *Salmonella* and *Shigella* isolates obtained from clinical specimens in Thailand.

2. Materials and methods

2.1. Shigella and Salmonella strains

Clinical isolates of 893 *Salmonella* and 253 *Shigella* from intestinal infection cases were obtained from The WHO National *Salmonella* and *Shigella* Centre in Thailand from 2011 to 2018. The WHO National *Salmonella* and *Shigella* Centre received all presumptive *Shigella* and *Salmonella* isolates from all public health laboratories in all regions of Thailand. All clinical isolates were tested for antimicrobial drug susceptibility to ESCs and quinolones. Cefotaxime-resistant clinical isolates were selected and tested in this study, which included 33 *Salmonella* spp. (consisting of 6 *Salmonella enterica* serovar Agona isolates, 25 *Salmonella enterica* serovar 4,5,12:i:- isolates, and 2 *Salmonella enterica* serovar Rissen isolates) and 53 *Shigella* spp. (consisting of 14 *Shigella sonnei* phase I isolates, 37 *Shigella sonnei* phase II isolates, 1 *Shigella sonnei* phase I, II isolate and 1 *Shigella flexneri* type 2a isolate). All isolates were confirmed for the included species using biochemical tests according to the method of Edwards and Ewing (1986). *Salmonella* isolates were serotyped according to antigenic characterization based on the White-Kaufmann-Le Minor scheme described by Popoff (2001). *Shigella* serotype was identified using specific antisera with slide agglutination according to the method of Ewing and Lindberg (1984).

2.2. Antimicrobial susceptibility and extended-spectrum beta-lactamase assay

Disk diffusion tests were performed according to the Clinical and Laboratory Standards Institute (CLSI) criteria (CLSI, 2018) using disks (Oxoid Limited, Hampshire, England) impregnated with ampicillin (AMP; 10 μg), cefotaxime (CTX; 30 μg), ceftriaxone (CRO; 30 μg), ceftazidime (CAZ; 30 μg), cefoxitin (FOX, 30 μg), ciprofloxacin (CIP; 5 μg), and nalidixic acid (NAL; 30 μg). The cultures were grown on Mueller-Hinton agar (Oxoid Limited, Hampshire, England), and zones of growth inhibition were measured following incubation at 37 °C for 24 h. *Escherichia coli* ATCC 25922 was used as a quality control and tested under the same conditions. The CLSI for "other Enterobacteriaceae" described a method for screening ESBL-producing strains using double-disk diffusion. Double-disk diffusion was performed using both cefotaxime (30 μg) and ceftazidime (30 μg) disks each alone and in combination with clavulanic acid (cefotaxime/clavulanic acid (30/10 μg) and ceftazidime/clavulanic acid (30/10 μg) (Oxoid Limited, Hampshire, England). *Klebsiella pneumoniae* ATCC 700603 (ESBL positive) and *E. coli* ATCC 25922 (ESBL negative) were used as quality control strains for ESBL screening test. The MICs for ceftazidime (256–0.016 μg/mL), ceftriaxone (256–0.016 μg/mL), cefotaxime (256–0.016 μg/mL), cefoxitin (256–0.016 μg/mL) and ciprofloxacin (32–0.002 μg/mL) were tested using the Liofilchem MIC Test Strips (Liofilchem, Roseto degli Abruzzi, Italy) following the instructions provided by the manufacturer.

2.3. PCR-based characterization of antimicrobial resistance genes

Antimicrobial resistance genes were detected using PCR and DNA sequencing. The primers and amplification conditions for each PCR reaction are listed in Table 1. Detection of the ESBL genes associated with β-lactams and cephalosporins-resistant *Shigella* and *Salmonella* was performed as previously described (Le et al., 2015; Ji et al., 2010; Hasman et al., 2005; Kojima et al., 2005; Olesen et al., 2004), and the CTX group, including the $bla_{\text{CTX-M-1}}$ group and $bla_{\text{CTX-M-9}}$ group primers, was confirmed as previously described (Le et al., 2015; Ji et al., 2010). Detection of mutations in the QRDR of *gyrA* and *parC* genes and screening for PMQR, including, *qnrA*, *qnrB*, *qnrS1*, and *aac(6')-lb-cr* genes, were achieved by PCR and sequencing as previously described (Pu et al., 2009; Cattoir et al., 2007; Hu et al., 2007; Park et al., 2006; Robicsek et al., 2006; Wiuff et al., 2000).

Genomic DNA from a pure culture grown at 37 °C on tryptic soy agar was extracted using the Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA). To detect resistance genes, Ex *Taq* DNA polymerase (Takara Bio Inc., Shiga, Japan) was used. The amplification conditions for all PCR reactions were standardized as follows: predenaturation at 95 °C for 5 min, followed by 30 cycles of denaturation at 98 °C for 10 s, annealing at optimum temperature for 1 min and DNA extension at 72 °C for 1–1.30 min, and a final extension at 72 °C for 10 min. The annealing temperatures varied for the different primers. The PCR products were analysed by gel electrophoresis using a 1% agarose gel run at 100 V for 30 min. To visualize band migration, the gel was stained with fluorescent dye and visualized under UV transillumination. A 1-kb or 100-bp ladder (Gibco BRL, Ontario, USA) was used to estimate the amplicon size. PCR amplicon product using primers specific to antimicrobial resistance genes were sequenced at the Macrogen company sequencing facility using an Applied Biosystems 3730 mXL automated DNA sequencer (Macrogen, Seoul, Korea). The DNA sequences were compared to those in the GenBank database (https://www.ncbi.nlm.nih.gov/) using the BLAST suite of sequence similarity searching programs.

3. Results

During the period of the study, 893 *Salmonella* and 253 *Shigella* stock cultures were chosen for antimicrobial drug susceptibility testing. The antimicrobial resistance patterns of clinical 53 *Shigella* and 33 *Salmonella* isolates against 7 drugs (ampicillin, ceftriaxone, cefotaxime, ceftazidime, cefoxitin, ciprofloxacin and nalidixic acid) were determined (Table 2 and Table 3). The results showed that all *Shigella* isolates were resistant to ampicillin, cefotaxime and ceftriaxone, followed by ceftazidime (84.9%), nalidixic acid (62.3%), ciprofloxacin (7.6%) and cefoxitin (1.9%) (Table 3). Four *Shigella sonnei* (7.5%) exhibited co-resistance to both ciprofloxacin and third-generation cephalosporins. Most of the

Table 1. Primers used in this study for determination ESBL, QDRD and PMQR genes of *Salmonella and Shigella.*

Primer	Sequence (5′ to 3′)	$T_{Anneal.}$ (°C)	Size (bp)	Ref.
ESBL and AmpC β-lactamases genes for *Salmonella*				
bla_{TEM}	F: GCGGAACCCCTATTTG R: ACCAATGCTTAATCAGTGAG	50	964	Olesen et al. (2004)
bla_{SHV}	F: TTCGCCTGTGTATTATCTCCCTG R: TTAGCGTTGCCAGTGYTCG	55	854	Hasman et al. (2005)
bla_{CMY-2}	F: GCACTTAGCCACCTATACGGCAG R: GCTTTTCAAGAATGCGCCAGG	55	758	Hasman et al. (2005)
$bla_{CTX-M-1}$ gr.	F: GAATTAGAGCGGCAGTCGGG R: CACAACCCAGGAAGCAGGC	56	588	Le et al. (2015)
$bla_{CTX-M-9}$ gr.	F: GTGCAACGGATGATGTTCGC R: GAAACGTCTCATCGCCGATC	56	475	Le et al. (2015)
ESBL and AmpC β-lactamases genes for *Shigella*				
bla_{TEM}	F: ATAAAATTCTTGAAGACGAAA R: GACAGTTACCAATGCTTAATC	45	1,076	Ji et al. (2010)
bla_{SHV}	F: GCCTTTTCGGCCTTCACTCAAG R: TTAGCGTTGCCAGTGCTCGATCA	55	989	Ji et al. (2010)
bla_{CMY-2}	F: ATGATGAAAAAATCGTTATGCT R: TTATTGCAGCTTTTCAAGAATGCG	60	1,122	Kojima et al. (2005)
$bla_{CTX-M-1}$ gr.	F: GGCCCATGGTTAAAAAATCACTGC R: CCGTTTCCGCTATTACAAACCGTTG	55	891	Ji et al. (2010)
$bla_{CTX-M-9}$ gr.	F: GTGACAAAGAGAGTGCAACGG R: TGATTCTCGCCGCTGAAGCC	55	856	Ji et al. (2010)
QRDR genes for *Salmonella*				
gyrA	F: TACCGTCATAGTTATCCACGA R: GTACTTTACGCCATGAACGT	56	588	Wiuff et al. (2000)
parC	F: CTATGCGATGTCAGAGCTGG R: TAACAGCAGCTCGGCCTATT	56	475	Wiuff et al. (2000)
PMQR genes for *Salmonella*				
qnrA	F: GGGTATGGATATTATTGATAAAG R: CTAATCCGGCAGCACTATTA	55	579	Cattoir et al. (2007)
qnrB	F: GGMATHGAAATTCGCCACTG R: TTTGCYGYYCGCCAGTCGAA	50	263	Cattoir et al. (2007)
qnrS	F: GCAAGTTCATTGAACAGGGT R: TCTAAACCGTCGAGTTCGGCG	55	427	Cattoir et al. (2007)
aac(6′)-lb-cr	F: TTGCGATGCTCTATGAGTGGCTA R: CTCGAATGCCTGGCGTGTTT	55	482	Park et al. (2006)
QRDR genes for *Shigella*				
gyrA	F: TACACCGGTCAACATTGAGG R: TTAATGATTGCCGCCGTCGG	52	648	Hu et al. (2007)
parC	F: GTACGTGATCATGGACCGTG R: TTCGGCTGGTCGATTAATGC	52	531	Hu et al. (2007)
PMQR for *Shigella*				
qnrA	F: ATTTCTCACGCCAGGATTTG R: GATCGGCAAAGGTYAGGTCA	55	579	Robicsek et al. (2006)
qnrB	F: GATCGTGAAAGCCAGAAAGG R: ACGAYGCCTGGTAGTTGTCC	53	263	Robicsek et al. (2006)
qnrS	F: TGGAAACCTACAATCATACA R: TGCAATTTTGATACCTGATG	46	427	Cano et al., 2009
aac(6′)-lb-cr	F: GCAACGCAAAAACAAAGTTAGG R: GTGTTTGAACCATGTACA	46	560	Pu et al., (2009)

$T_{Anneal.}$: Annealing temperature.

Salmonella isolates (consisting of 25 isolates of *Salmonella* serovar 4,5,12:i:-, 6 isolates of *Salmonella* serovar Agona and 2 isolates of *Salmonella* serovar Rissen) were 100% resistant to ampicillin, cefotaxime, ceftazidime and ceftriaxone. Seven (21.2%) *Salmonella* isolates (consisting of 5 isolates of *Salmonella* serovar Agona and 2 isolates of *Salmonella* serovar Rissen) showed resistance to cefoxitin (Table 2). Ten (30.3%) isolates (consisting of 6 isolates of *Salmonella* serovar 4,5,12:i:-, 2 of isolates *Salmonella* serovar Agona and 2 of isolates *Salmonella* serovar Rissen) showed co-resistance to ciprofloxacin and cefotaxime.

Table 2. Antimicrobial susceptibility and ESBL-producing of *Shigella* isolates from clinical specimens.

Antimicrobial agent (μg)	Isolates (%)		
	R/ESBL	I/ESBL	S/ESBL
AMP (10)	53 (100.0)/ 52 (98.1)	-	-
FOX (30)	1 (1.9)/ 1 (1.9)	-	52 (98.1)/ 51 (96.2)
CTX (30)	53 (100.0)/ 52 (98.1)	-	-
CAZ (30)	45 (84.9)/ 45 (84.9)	6 (11.3)/ 6 (11.3)	2 (3.8)/ 1 (1.9)
CRO (30)	53 (100.0)/ 52 (98.1)	-	-
NAL (30)	33 (62.3)/ 31 (58.5)	16 (30.2)/ 16 (30.2)	4 (7.6)/ 3 (5.7)
CIP (5)	4 (7.6)/ 4 (7.6)	40 (75.5)/ 38 (71.7)	9 (17.0)/ 8 (15.1)

S, susceptibility; I, intermediate; R resistant; AMP, ampicillin; CAZ, ceftazidime; CTX, cefotaxime; CRO, ceftriaxone; FOX, cefoxitin; NAL, nalidixic acid and CIP, ciprofloxacin.

Fifty-two (98.1%) and thirty-two (97.0%) cefotaxime-resistant *Shigella* and *Salmonella* isolates, respectively, were positive for ESBL production. The ESBL-producing strains screened in the present study were resistant to two classes of antibiotics, including quinolones and third-generation cephalosporins (antimicrobials used to treat bacterial infections) (Table 4). The strains with the highest multidrug resistance were 2 isolates of *Salmonella* serovar Agona, 2 isolates of *Salmonella* serovar Rissen, and 1 isolate of *Shigella sonnei*, which were resistant to seven antimicrobial agents (AMP-FOX-CTX-CAZ-CRO-CIP-NAL). All ESBL-producing *Salmonella* isolates were resistant to cefotaxime and ceftriaxone in 100% of the isolates, and cross-resistance to cefoxitin in 5 (15.1%) *Salmonella* serovar Agona isolates and 2 (6.1%) *Salmonella* serovar Rissen isolates. Additionally, all ESBL-producing *Shigella* isolates were resistant to cefotaxime and ceftriaxone in 100% of the isolates and cross-resistance to ceftazidime and cefoxitin in 46 (86.8%) and 1 (1.9%) of the *Shigella sonnei* isolates, respectively.

Table 3. Antimicrobial susceptibility and ESBL-producing of *Salmonella* isolates from clinical specimens.

Antimicrobial agent (μg)	Number of isolates (%)		
	R/ESBL	I/ESBL	S/ESBL
AMP (10)	33 (100)/ 32 (97.0)	-	-
FOX (30)	7 (21.2)/ 7 (21.2)	5 (15.2)/ 5 (15.2)	21 (63.6)/ 20 (60.6)
CTX (30)	33 (100.0)/ 32 (97.0)	-	-
CAZ (30)	32 (100.0)/ 32 (100.0)	-	1 (3.03)/ 0 (0.0)
CRO (30)	33 (100.0)/ 32 (97.0)	-	-
NAL (30)	10 (30.3)/ 10 (30.3)	9 (27.3)/ 8 (24.2)	14 (42.4)/ 14 (42.4)
CIP (5)	10 (30.3)/ 10 (30.3)	16 (48.5)/ 15 (45.4)	7 (21.2)/ 7 (21.2)

S, susceptibility; I, intermediate; R resistant; AMP, ampicillin; CAZ, ceftazidime; CTX, cefotaxime; CRO, ceftriaxone; FOX, cefoxitin; NAL, nalidixic acid and CIP, ciprofloxacin.

Table 4. Antimicrobial patterns and ESBL/non ESBL-producing of *Salmonella* and *Shigella* isolates from clinical specimens.

Strain	Antimicrobial resistance pattern	No. of isolates	
		ESBL (%)	Non-ESBL (%)
Sal. serovar,			
S. Agona	AMP-CTX-CAZ-CRO	1 (3.0)	0 (0.0)
	AMP-FOX-CTX-CAZ-CRO	3 (9.1)	0 (0.0)
	AMP-FOX-CTX-CAZ-CRO-CIP-NAL	2 (6.1)	0 (0.0)
S.I. 4,5,12:i:-	AMP-CTX-CRO	0 (0.0)	1 (3.0)
	AMP-CTX-CAZ-CRO	18 (54.5)	0 (0.0)
	AMP-CTX-CAZ-CRO-CIP-NAL	6 (18.2)	0 (0.0)
S. Rissen	AMP-FOX-CTX-CAZ-CRO-CIP-NAL	2 (6.1)	0 (0.0)
Total		32	1
Shi. serotype,			
Sonnei	AMP-CTX-CRO	3 (5.7)	0 (0.0)
	AMP-CTX-CAZ-CRO	17 (32.1)	0 (0.0)
	AMP-CTX-CRO-NAL	2 (3.8)	1 (1.1)
	AMP-CTX-CAZ-CRO-NAL	25 (47.2)	0 (0.0)
	AMP-CTX-CAZ-CRO-CIP-NAL	3 (5.7)	0 (0.0)
	AMP-FOX-CTX-CAZ-CRO-CIP-NAL	1 (1.9)	0 (0.0)
Flexneri	AMP-CTX-CRO-NAL	1 (1.9)	0 (0.0)
Total		52	1

AMP, ampicillin; CAZ, ceftazidime; CTX, cefotaxime; CRO, ceftriaxone; FOX, cefoxitin; NAL, nalidixic acid and CIP, ciprofloxacin.

The present study provides the first description of the variety of the β-lactamase resistance gene patterns in *Salmonella* and *Shigella* isolates from clinical samples in Thailand (Table 5 and Table 6). The results showed that β-lactamase genes encoding CMY-2, TEM-1 and CTX-M were found in 26, 21, and 19 ESBL-producing *Salmonella* isolates, respectively. Based on PCR assays and sequencing, the CTX-M-1 group was more prevalent than the CTX-M-9 group. The CTX-M-1 and CTX-M-9 groups were represented by $bla_{\text{CTX-M-15}}$ genes (18 isolates) and $bla_{\text{CTX-M-14}}$ genes (1 isolate), respectively. In addition, 39, 34 and 22 of ESBL-producing *Shigella* isolates harboured β-lactamase resistance gene encoding CTX-M, TEM-1 and CMY-2, respectively. ESBL-producing *Shigella* isolates carrying $bla_{\text{CTX-M-15}}$ genes (35 isolates) were significantly higher than those carrying $bla_{\text{CTX-M-14}}$ genes (4 isolates). Thirty-two ESBL-producing *Salmonella* showed high MIC levels to cefotaxime (96–≥256 μg/ml), ceftazidime (32–≥256 μg/ml), ceftriaxone (≥256 μg/ml) and cefoxitin (1.5–≥256 μg/ml). Fifty-two ESBL-producing *Shigella sonnei* isolates exhibited high MIC levels to cefotaxime (94–≥256 μg/ml), ceftazidime (12–≥256 μg/ml), ceftriaxone (128–≥ 256 μg/ml) and cefoxitin (1.5–8 μg/ml). Three *Shigella sonnei* isolates showed high MIC levels ≥256 μg/ml to cefotaxime, ceftazidime and ceftriaxone.

As shown in Table 7, different of β-lactamase gene patterns were detected in ESBL-producing ciprofloxacin-resistant *Shigella* and *Salmonella* isolates. Among the fourteen ESBL-producing cefotaxime and ciprofloxacin co-resistant isolates, 6 isolates of *Salmonella* serovar 4,5,12:i:- harboured $bla_{\text{CTX-M-15}}$, $bla_{\text{TEM-1}}$, $bla_{\text{CMY-2}}$ (5 isolates) and $bla_{\text{CTX-M-15}}$, $bla_{\text{TEM-1}}$ (1 isolate); 2 isolate of *Salmonella* serovar Agona harboured the $bla_{\text{CTX-M-15}}$, $bla_{\text{TEM-1}}$, $bla_{\text{CMY-2}}$ (1 isolate) and $bla_{\text{TEM-1}}$, $bla_{\text{CMY-2}}$ (1 isolate);

Table 5. MIC values to cephalosporins and ESBL genes of 32 ESBL-producing of *Salmonella* isolates from clinical specimens.

Strain	Antimicrobial pattern	No. isolate	MIC (μg/ml)				β-lactamase and ESBL gene
			FOX	CTX	CAZ	CRO	
S. Agona	FOX-CTX-CAZ-CRO-CIP-NAL (n = 2)	1	≥256	128	≥256	≥256	$bla_{\text{CTX-M-15}}$, $bla_{\text{TEM-1}}$, $bla_{\text{CMY-2}}$
		1	≥256	96	≥256	≥256	$bla_{\text{TEM-1}}$, $bla_{\text{CMY-2}}$
	FOX-CTX-CAZ-CRO (n = 3)	1	≥256	96	≥256	≥256	$bla_{\text{CTX-M-15}}$, $bla_{\text{TEM-1}}$, $bla_{\text{CMY-2}}$
		1	≥256	128	96	≥256	$bla_{\text{TEM-1}}$, $bla_{\text{CMY-2}}$
		1	96	≥256	128	≥256	$bla_{\text{TEM-1}}$, $bla_{\text{CMY-2}}$
	CTX-CAZ-CRO (n = 1)	1	4	128	≥256	≥256	$bla_{\text{TEM-1}}$, $bla_{\text{CMY-2}}$
*S.*I. 4,5,12:I: -	CTX-CAZ-CRO-CIP-NAL (n = 6)	1	12	≥256	32	≥256	$bla_{\text{CTX-M-15}}$, $bla_{\text{TEM-1}}$, $bla_{\text{CMY-2}}$
		3	6	≥256	32	≥256	$bla_{\text{CTX-M-15}}$, $bla_{\text{TEM-1}}$, $bla_{\text{CMY-2}}$
		1	2	≥256	192	≥256	$bla_{\text{CTX-M-15}}$, $bla_{\text{TEM-1}}$, $bla_{\text{CMY-2}}$
		1	2	≥256	96	≥256	$bla_{\text{CTX-M-15}}$, $bla_{\text{TEM-1}}$
	CTX-CAZ-CRO (n = 18)	1	4	≥256	48	≥256	$bla_{\text{CTX-M-15}}$, $bla_{\text{TEM-1}}$, $bla_{\text{CMY-2}}$
		1	4	≥256	96	≥256	$bla_{\text{CTX-M-15}}$, $bla_{\text{TEM-1}}$, $bla_{\text{CMY-2}}$
		2	2	≥256	32	≥256	$bla_{\text{CTX-M-15}}$, $bla_{\text{TEM-1}}$, $bla_{\text{CMY-2}}$
		1	3	≥256	64	≥256	$bla_{\text{CTX-M-15}}$, $bla_{\text{TEM-1}}$, $bla_{\text{CMY-2}}$
		1	2	≥256	192	≥256	$bla_{\text{CTX-M-15}}$, $bla_{\text{TEM-1}}$, $bla_{\text{CMY-2}}$
		1	12	≥256	32	≥256	$bla_{\text{CTX-M-15}}$, $bla_{\text{CMY-2}}$
		1	16	≥256	48	≥256	$bla_{\text{CTX-M-15}}$, $bla_{\text{CMY-2}}$
		1	6	≥256	32	≥256	$bla_{\text{CTX-M-15}}$, $bla_{\text{CMY-2}}$
		1	2	≥256	128	≥256	$bla_{\text{CTX-M-15}}$, $bla_{\text{CMY-2}}$
		1	1.5	≥256	64	≥256	$bla_{\text{CTX-M-15}}$, $bla_{\text{CMY-2}}$
		1	2	≥256	64	≥256	$bla_{\text{CTX-M-15}}$, $bla_{\text{TEM-1}}$
		1	8	≥256	32	≥256	$bla_{\text{CTX-M-14}}$, $bla_{\text{TEM-1}}$, $bla_{\text{CMY-2}}$
		1	3	≥256	64	≥256	$bla_{\text{TEM-1}}$, $bla_{\text{CMY-2}}$
		1	12	128	64	≥256	$bla_{\text{TEM-1}}$, $bla_{\text{CMY-2}}$
		1	12	≥256	32	≥256	$bla_{\text{TEM-1}}$, $bla_{\text{CMY-2}}$
		1	1.5	≥256	≥256	≥256	$bla_{\text{CMY-2}}$
		1	2	≥256	64	≥256	$bla_{\text{CMY-2}}$
S. Rissen	FOX-CTX-CAZ-CRO-CIP-NAL (n = 2)	1	192	≥256	64	≥256	$bla_{\text{CTX-M-15}}$, $bla_{\text{TEM-1}}$
		1	≥256	96	≥256	≥256	$bla_{\text{CMY-2}}$

CAZ, ceftazidime; CTX, cefotaxime; CRO, ceftriaxone; FOX, cefoxitin, NAL, nalidixic acid and CIP, ciprofloxacin.

Table 6. MIC values to cephalosporins and β-lactamase genes of 52 ESBL-producing *Shigella* isolates from clinical specimens.

Strain	Antimicrobial pattern	No. isolate	MIC (μg/ml)				β-lactamase and ESBL gene
			FOX	CTX	CAZ	CRO	
S. sonnei phase I	CTX-CAZ-CRO-CIP-NAL (n = 1)	1	6	≥256	192	≥256	$bla_{CTX-M-15}$, bla_{TEM-1}, bla_{CMY-2}
	CTX-CAZ-CRO-NAL (n = 5)	2	2	≥256	64	≥256	$bla_{CTX-M-15}$, bla_{TEM-1}, bla_{CMY-2}
		1	1.5	128	32	≥256	$bla_{CTX-M-15}$, bla_{TEM-1}
		1	3	≥256	48	≥256	$bla_{CTX-M-15}$, bla_{CMY-2}
		1	1.5	≥256	32	192	$bla_{CTX-M-14}$, bla_{TEM-1}
	CTX-CAZ-CRO (n = 6)	2	3	≥256	48	≥256	$bla_{CTX-M-15}$, bla_{TEM-1}, bla_{CMY-2}
		1	3	128	32	≥256	$bla_{CTX-M-15}$, bla_{TEM-1}
		1	2	≥256	32	≥256	bla_{TEM-1}, bla_{CMY-2}
		1	2	128	32	≥256	$bla_{CTX-M-15}$, bla_{TEM-1}
		1	2	≥256	48	≥256	$bla_{CTX-M-15}$
	CTX-CRO-NAL (n = 1)	1	2	94	16	≥256	$bla_{CTX-M-15}$, bla_{TEM-1}, bla_{CMY-2}
	CTX-CRO (n = 1)	1	2	≥256	12	≥256	$bla_{CTX-M-15}$, bla_{TEM-1}, bla_{CMY-2}
S. sonnei phase II	CTX-CAZ-CRO-CIP-NAL (n = 2)	1	6	≥256	≥256	≥256	$bla_{CTX-M-15}$, bla_{TEM-1}, bla_{CMY-2}
		1	6	≥256	128	≥256	$bla_{CTX-M-15}$, bla_{TEM-1}, bla_{CMY-2}
	CTX-CAZ-CRO-NAL (n = 20)	5	4	≥256	48	≥256	$bla_{CTX-M-15}$, bla_{TEM-1}, bla_{CMY-2}
		1	3	≥256	48	≥256	$bla_{CTX-M-15}$, bla_{TEM-1}, bla_{CMY-2}
		1	6	≥256	≥256	≥256	$bla_{CTX-M-15}$, bla_{TEM-1}, bla_{CMY-2}
		1	4	≥256	32	128	$bla_{CTX-M-15}$, bla_{TEM-1}, bla_{CMY-2}
		1	4	≥256	≥256	≥256	$bla_{CTX-M-15}$, bla_{TEM-1}, bla_{CMY-2}
		1	3	96	32	≥256	$bla_{CTX-M-15}$, bla_{TEM-1}
		1	2	≥256	48	≥256	$bla_{CTX-M-15}$, bla_{TEM-1}
		1	2	192	32	≥256	$bla_{CTX-M-15}$, bla_{TEM-1}
		1	3	≥256	32	≥256	$bla_{CTX-M-15}$, bla_{TEM-1}
		1	3	≥256	64	≥256	$bla_{CTX-M-15}$, bla_{CMY-2}
		1	3	≥256	48	≥256	bla_{TEM-1}, bla_{CMY-2}
		1	4	≥256	96	≥256	bla_{TEM-1}, bla_{CMY-2}
		3	3	≥256	48	≥256	$bla_{CTX-M-15}$
		1	3	≥256	32	≥256	$bla_{CTX-M-14}$, bla_{TEM-1}
	CTX-CAZ-CRO (n = 11)	1	8	≥256	32	≥256	$bla_{CTX-M-15}$, bla_{TEM-1}, bla_{CMY-2}
		1	6	≥256	32	≥256	$bla_{CTX-M-15}$, bla_{TEM-1}
		2	3	≥256	32	≥256	$bla_{CTX-M-15}$, bla_{TEM-1}
		1	1.5	≥256	32	≥256	$bla_{CTX-M-15}$, bla_{TEM-1}
		2	3	≥256	32	≥256	$bla_{CTX-M-15}$, bla_{TEM-1}
		1	3	≥256	48	≥256	$bla_{CTX-M-15}$, bla_{CMY-2}
		1	3	≥256	48	≥256	$bla_{CTX-M-15}$
		1	4	≥256	32	≥256	$bla_{CTX-M-14}$, bla_{TEM-1}, bla_{CMY-2}
		1	3	≥256	32	≥256	$bla_{CTX-M-14}$
	CTX-CRO-NAL (n = 1)	1	3	96	24	≥256	$bla_{CTX-M-15}$, bla_{TEM-1}
	CTX-CRO (n = 2)	1	3	≥256	24	≥256	$bla_{CTX-M-15}$, bla_{TEM-1}
		1	3	≥256	16	≥256	$bla_{CTX-M-15}$, bla_{TEM-1}, bla_{CMY-2}
S. sonnei phase I,II	FOX-CTX-CAZ-CRO-CIP-NAL (n = 1)	1	≥256	≥256	≥256	128	$bla_{CTX-M-15}$, bla_{CMY-2}
S. flexneri type 2a	CTX-CRO-NAL (n = 1)	1	6	48	1.5	≥256	$bla_{CTX-M-15}$, bla_{TEM-1}

CAZ, ceftazidime; CTX, cefotaxime; CRO, ceftriaxone; FOX, cefoxitin, NAL, nalidixic acid and CIP, ciprofloxacin.

and *Shigella sonnei* harboured ESBLs combined with AmpC β-lactamase genes containing $bla_{CTX-M-15}$, bla_{TEM-1}, bla_{CMY-2} (3 isolates) and $bla_{CTX-M-15}$, bla_{CMY-2} (1 isolate).

Ten isolates of cefotaxime and ciprofloxacin co-resistant *Salmonella* were ESBL producers, and we observed QRDR mutations in the *gyrA* (at S83F, D87G and D87Y) and *parC* (at T57S) genes, while point mutations in *gyrA* (at S83L and D87Y or D87G) and no mutation in *parC* were detected in three cefotaxime and ciprofloxacin co-resistant *Shigella sonnei* isolates. Only one *Shigella sonnei* phase I isolate showed a point mutation in the *gyrA* gene at S83L and D87Y and the *parC* gene at S80I. Ten cefotaxime and ciprofloxacin co-resistant *Salmonella* isolates showed 5 patterns of PMQR genes, including *aac(6′)-lb-cr* (4 isolates), *qnrB* and *aac(6′)-lb-cr* (1 isolate), *qnrB* and *qnrS1* (2 isolates), *qnrB* (2 isolates), and *qnrS1* (1 isolate). This finding showed that the plasmid harbouring the *qnrS* gene was present in all co-resistant *Shigella sonnei* isolated from the clinical sample.

Among the co-resistant isolates, the highest MIC level for ciprofloxacin (1 μg/ml) was observed for each isolate of *Salmonella* serovar Agona, *Salmonella* serovar 4,5,12:i:- and *Shigella sonnei* isolated from clinical specimens, and *Salmonella* harboured a mutation in *gyrA* (at S83F and D87G) and *parC* (at T57S) combined with *aac(6′)-lb-cr*, and *Shigella sonnei* harboured a mutation in *gyrA* (at S83L and D87Y) and *parC* (at S80I) combined with *qnrS1*.

4. Discussion

According to the WHO National *Salmonella* and *Shigella* Centre annual report, 5,401 non-typhoidal *Salmonella* isolates and 253 *Shigella* isolates

Table 7. Genotypic features of the ESBL-producing ciprofloxacin-resistant *Salmonella and Shigella* isolates.

ID	Source	Sex/Age	Strain	β-lactamase and ESBL gene	MIC CIP	QRDR mutation:		PMQR gene
					(μg/ml)	*gyrA*	*parC*	
SH 968	Blood	F/75 y	*S.* Agona	$bla_{CTX\text{-}M\text{-}15}$, $bla_{TEM\text{-}1}$, $bla_{CMY\text{-}2}$	1	S83F, D87G	T57S	*aac-(6′)-lb*
SH 1902	Stool	F/87 y	*S.* Agona	$bla_{TEM\text{-}1}$, $bla_{CMY\text{-}2}$	0.5	S83F, D87Y	T57S	*aac-(6′)-lb*
SH 713	Stool	M/1 y	*S.*I. 4,5,12:i:-	$bla_{CTX\text{-}M\text{-}15}$, $bla_{TEM\text{-}1}$, $bla_{CMY\text{-}2}$	0.064	WT	WT	*qnrB*
SH 714	Stool	F/55 y	*S.*I. 4,5,12:i:-	$bla_{CTX\text{-}M\text{-}15}$, $bla_{TEM\text{-}1}$, $bla_{CMY\text{-}2}$	0.047	WT	WT	*qnrS, qnrB*
SH 1075	Urine	F/59 y	*S.*I. 4,5,12:i:-	$bla_{CTX\text{-}M\text{-}15}$, $bla_{TEM\text{-}1}$, $bla_{CMY\text{-}2}$	0.047	WT	WT	*aac-(6′)-lb,qnrB*
SH 1123	Stool	M/51 y	*S.*I. 4,5,12:i:-	$bla_{CTX\text{-}M\text{-}15}$, $bla_{TEM\text{-}1}$, $bla_{CMY\text{-}2}$	0.75	WT	WT	*aac-(6′)-lb*
SH 1337	Rectal swab	F/43 y	*S.*I. 4,5,12:i:-	$bla_{CTX\text{-}M\text{-}15}$, $bla_{TEM\text{-}1}$, $bla_{CMY\text{-}2}$	0.047	WT	WT	*qnrB*
SH 1756	Rectal swab	F/15 y	*S.*I. 4,5,12:i:-	$bla_{CTX\text{-}M\text{-}15}$, $bla_{TEM\text{-}1}$	1	S83F, D87G	WT	*aac-(6′)-lb*
SH 1493	Stool	M/3 y	*S.* Rissen	$bla_{CMY\text{-}2}$	0.38	WT	WT	*qnrS*
SH 1761	Stool	F/81 y	*S.* Rissen	$bla_{CTX\text{-}M\text{-}15}$, $bla_{TEM\text{-}1}$	0.5	S83F	T57S	*qnrS, qnrB*
Shi 235	Rectal swab	F/32 y	*Shi. sonnei* I	$bla_{CTX\text{-}M\text{-}15}$, $bla_{TEM\text{-}1}$, $bla_{CMY\text{-}2}$	1	S83L, D87Y	S80I	*qnrS*
Shi 244	Stool	M/11 y	*Shi. sonnei* II	$bla_{CTX\text{-}M\text{-}15}$, $bla_{TEM\text{-}1}$, $bla_{CMY\text{-}2}$	0.5	S83L, D87Y	WT	*qnrS*
Shi 274	Stool	M/1 y	*Shi. sonnei* II	$bla_{CTX\text{-}M\text{-}15}$, $bla_{TEM\text{-}1}$, $bla_{CMY\text{-}2}$	0.75	S83L, D87Y	WT	*qnrS*
Shi 15	Rectal swab	F/6 y	*Shi. sonnei* I,II	$bla_{CTX\text{-}M\text{-}15}$, $bla_{CMY\text{-}2}$	0.5	S83L, D87G	WT	*qnrS*

ESBL, extended-spectrum beta-lactamase; QRDR, quinolone-resistance determining region; PMQR, plasmid-mediated quinolone resistance; CIP, ciprofloxacin; WT, wild type; F, Phenylalanine; G, glycine; I, isoleucine; S, Serine; T, Threonine; Y, Tyrosine; M, Male; F, Female.

from 2011 to 2018 were confirmed serotype from pure culture isolated from patients in Thailand. The overall percentage of *Salmonella* serovar during 2015–2018 showed that the dominant *Salmonella* isolates were identified as *S*. Enteritidis (25.8%), followed by *S*. Choleraesuis (16.9%), *Salmonella enterica* serovar 4,5,12:i:- (12.2%), *S*. Weltevreden (7.2%) and *S*. Stanley (4.8%). A high proportion of *Shigella* infection cases was observed in 2011 (145 isolates). However, the number of *Shigella* cases decreased from 72 isolates in 2012 to 21 isolates in 2013, 11 isolates in 2014, 3 isolates in 2017 and 1 isolate in 2018. *Shigella sonnei* (71.9%) was the most prevalent serotype found in clinical samples.

Multidrug-resistant *Salmonella* and *Shigella* may pose a risk to humans, particularly individuals resistant to fluoro (quinolone) and third-generation cephalosporins drugs. The previous research studies in Thailand have described the presence of multidrug-resistant *Salmonella* and *Shigella* from a wide range of serovars and serotypes, respectively (Whistler et al., 2018; Sirichote et al., 2010; Chompook et al., 2005). Previous data showed that *Shigella sonnei* was the dominant *Shigella* species isolated from patients in Thailand (Chompook et al., 2005; Hiranrattana et al., 2005), and *Shigella sonnei* phases I and II were also commonly observed serotypes (Pulsrikarn et al., 2009). In developed countries, several previous reports have shown that *Shigella sonnei* is more prevalent other *Shigella* species (Kahsay and Muthupandian, 2016; Kotloff et al., 1999). The present study showed that all *Shigella sonnei* isolates were resistant to ceftriaxone and cefotaxime, and that 4 isolates (7.6%) were resistant to ciprofloxacin. The above findings are in contrast with antimicrobial susceptibility studies in Thailand, which reported that *Shigella sonnei* strains isolated from the stool culture specimens of children were susceptible to ceftriaxone (Chompook et al., 2005; Hiranrattana et al., 2005).

The present study indicated that 33 (100%), 33 (100%) and 10 (30.3%) *Salmonella* isolates were resistance against ampicillin, third-generation cephalosporins (cefotaxime and ceftriaxone) and ciprofloxacin, respectively. Our results agreed with a previous study in Thailand, ESBL-producing *S.* Choleraesuis isolates from patients with systemic infections were 100% resistant to ampicillin and ESCs, and 14.6% were resistant to ciprofloxacin (Sriyapai et al., 2021). The most important mechanism of resistance to a broad class of beta-lactam antibiotics (ampicillin and ESCs) involves the production of beta-lactamases (particularly ESBLs), which inactivate beta-lactam antibiotics. Lee et al. (2009) found a high level of resistance to ceftriaxone and ciprofloxacin among non-typhoidal *Salmonella* in Southeast Asian countries. Previous studies in Thailand and Taiwan observed the highest frequencies resistance to ciprofloxacin and ceftriaxone of clinical *Salmonella* (Kulwichit et al., 2007; Li et al., 2005). In our study, *Salmonella* (10 isolates) and *Shigella* (4 isolates) ESBL producers isolated from humans were co-resistant to third-generation cephalosporins (including ceftriaxone, cefotaxime and ceftazidime) and ciprofloxacin. Although the findings of this study do not indicate that *Salmonella* and *Shigella* are invasive serotypes, if resistance to ESCs and fluoroquinolone is acquired, then the treatment of these infections using β-lactam and fluoroquinolones antibiotics could be severely compromised. Third-generation cephalosporins (including cefotaxime or ceftriaxone) and fluoroquinolones (ciprofloxacin) are the standard drugs for treating *Salmonella* and *Shigella* infections in humans (Williams and Berkley, 2018; Whistler et al., 2018). This antimicrobial finding highlights a serious epidemiological situation.

In the present study, β-lactamase genes encoding CTX-M were detected in 39 (73.6%) isolates of *Shigella* spp. and 19 (57.6%) isolates of *Salmonella* spp., whereas the sequencing results showed that CTX-M isolates belonging to CTX-M-15 (35 *Shigella* and 18 *Salmonella* isolates) were higher than those belonging to CTX-M-14 (4 *Shigella* and 1 *Salmonella* isolates). The present study reports the description of ESBL producers of *Salmonella* and *Shigella* isolates in Thailand, in which dominant ESBL isolates harboured $bla_{CTX\text{-}M\text{-}15}$ with/without other ESBL resistance genes. This finding is similar to recently reported data from Iran (Abbasi et al., 2019; Bialvaei et al., 2017) and China (Guo and Zhao, 2021; Zhang et al., 2014) that showed the distribution of $bla_{CTX\text{-}M}$ genes in *Salmonella* and *Shigella* isolates.

According to various reports from around the world, CTX-M-1 is the most significant group presenting a risk to human health (Kim et al., 2016). These results showed high incidence of *Salmonella* and *Shigella* strains compete with *E. coli* and *Klebsiella* species as ESBL producers. Antimicrobial drug treatment enhances the prevalence of ESBL-producing strains by resistant strains selection (Spanu et al., 2002). Moreover, the horizontal gene transfer of antibiotic resistance genes (ARGs) of ESBL producers enhance the incidence of resistance to ESCs and other antimicrobial agents, such as aminoglycosides and fluoroquinolones (Laxminarayan et al., 2013).

In addition, ESBL-producing *Salmonella* and *Shigella* isolates were also resistant to ciprofloxacin. Two ESBL *Salmonella* isolates displayed QRDR mutations in the *gyrA* (S83F, D87G and D87Y) and *parC* (T57S) genes. The most commonly observed point mutations in the *gyrA* gene are the amino acid changes at codon serine-83 (serine to phenylalanine or tyrosine) and codon aspartic acid-87 (aspartic acid to glycine or asparagine or tyrosine), or/and mutations in *parC* involving changes at codon

threonine-57 (threonine to serine or tyrosine), which have been found in fluoroquinolone-resistant *Salmonella* (Cloeckaert et al., 2001). Three ESBL-producing, co-resistant *Shigella sonnei* only showed mutations in *gyrA* (S83L, D87Y and D87G), and these QRDR mutations have been implicated as being responsible for ciprofloxacin resistance in *Shigella sonnei* (Folster et al., 2011). The present study is the first published report on mutations in both *gyrA* (S83L and D87Y) and *parC* (S80I) genes in a single *Shigella sonnei* phase I isolate from patient in Thailand.

PMQR genes were observed in ESBL-producing ciprofloxacin resistance isolates. All of the co-resistant isolates harboured at least one of the PMQR gene including the *qnr* genes (*qnrB* and/or *qnrS*) and/or the *aac(6′)-Ib-cr* gene. To our knowledge, this study is the first report of QRDR mutations and *qnrS*-positive plasmids in clinical *Shigella sonnei* isolates in Thailand, and the results also showed an improved MIC of ciprofloxacin at 0.5–1 μg/ml. Low-level quinolone resistance is mediated by PMQR determinants, and the presence of PMQR genes is of great concern because these genes can promote mutations within the QRDR (Rodríguez-Martínez et al., 2016), in which *qnrS* was the commonly found allele of the *qnr* genes in *Shigella* spp. (Pu et al., 2015).

In conclusion, the current study showed a high incidence of ESBL producers among *Salmonella* and *Shigella* strains isolated from clinical specimens in Thailand. ESBL-producing isolates may pose public health risks. Notably, ESBL-producing *Salmonella* and *Shigella* isolated from clinical specimens were resistant to ESCs and fluoroquinolone. The present study provides the first description diversity of $bla_{\text{CTX-M-15}}$ plus other ESBL genes, as well as to identify QRDR mutations and PMQR genes in ESBL-producing *Salmonella* and *Shigella*. The marked diversity of antimicrobial resistance patterns and resistant gene patterns among the ESBL-producing isolates are associated with high MIC values.

Declarations

Author contribution statement

Thayat Sriyapai: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Chaiwat Pulsrikarn, Kosum Chansiri: Contributed reagents, materials, analysis tools or data.

Pichapak Sriyapai: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Data availability statement

Data included in article/supp. material/referenced in article.

Declaration of interest's statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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