Biocontrol Science, 2022, Vol. 27, No. 4, 209—215

Original

Simple and Rapid Detection of ESBL *bla*$_{SHV}$ gene from an Urban River in Tokyo by Loop-Mediated Isothermal Amplification

MASAHIKO OKAI[1], RINTARO ENDO[2], MASACHIKA TAKASHIO[3], MASAMI ISHIDA[1], AND NAOTO URANO[1]*

[1]*Department of Ocean Sciences, Tokyo University of Marine Science and Technology, 4-5-7 Konan, Minato-ku, Tokyo 108-8477, Japan*
[2]*Graduate School of Marine Science and Technology, Tokyo University of Marine Science and Technology, 4-5-7 Konan, Minato-ku, Tokyo 108-8477, Japan*
[3]*Zensho Laboratories of Food Technology, Zensho Holdings Co., Ltd., 2-18-1 Konan, Minato-ku, Tokyo 108-0075, Japan*

Received 8 June, 2022/Accepted 18 September, 2022

Extended-spectrum β-lactamases (ESBLs) are produced mainly by gram-negative bacteria in Enterobacteriaceae. One of the major types of ESBLs is sulfhydryl variable (SHV)-type ESBL. Herein, we attempted to develop a simple and rapid method for the detection of the ESBL *bla*$_{SHV}$ gene by loop-mediated isothermal amplification (LAMP). The five-primer set designed could amplify *bla*$_{SHV}$ gene at an isothermal temperature of 65℃. The detection limit of the LAMP method with the LF loop primer was 1 copy/tube, which was 10,000-fold more sensitive than that of the conventional PCR. The LAMP assay could also detect the direct amplification of *bla*$_{SHV}$ gene from a single river water sample in Tokyo. The LAMP method has great potential for applications in hospital, soil and water environment, food, and livestock.

Key words : *bla*$_{SHV}$ gene / *Escherichia coli* / Extended-spectrum β-lactamase / *Klebsiella pneumoniae* / Loop-mediated isothermal amplification.

INTRODUCTION

The broad overuse of β-lactam antibiotics against pathogens has caused the occurrence of extended-spectrum β-lactamase (ESBL)-producing bacteria. ESBLs, which hydrolyze expanded-spectrum β-lactam antibiotics including cephalosporin and monobactam, are a concern in both healthcare settings and the community. The ESBLs can be classified into the groups of Ambler's class A (TEM, SHV, CTX-M, GES, PER, VEB, and TLA-types) and class D (OXA-type) (Bradford, 2001). The major types of ESBLs are TEM, SHV, and CTX-M (Ghafourian et al., 2015).

The first sulfhydryl variable (SHV)-type ESBL was identified in a clinical isolate of *Klebsiella ozaenae* in Germany and designated as SHV-2 (Kliebe et al., 1985). SHV-2 differed from the well-known β-lactamase SHV-1 by only a single amino acid residue (Barthélémy et al., 1988). After the detection of SHV-2 in 1985, 184 SHV variants containing 45 SHV-type ESBLs have been annotated (https://www.ncbi.nlm.nih.gov/pathogens/refgene/#shv). The SHV-type ESBLs are typically encoded on transferable plasmids and among them, SHV-2, SHV-5, and SHV-12 have been prevalent in Enterobacteriaceae (Liakopoulos et al., 2016). Over the last two decades, host bacteria other than the typical ESBL-producing bacteria *Escherichia coli* (*E. coli*) and *Klebsiella pneumoniae* (*K. pneumoniae*) have also been described; e.g. *Pseudomonas aeruginosa* (Chen et al., 2015).

Loop-mediated isothermal amplification (LAMP) is a simple method that amplifies target DNA at isothermal temperature. The LAMP method is useful for the detection of bacterial and virus infections because it has high rapidity and sensitivity. There are reports of the detec-

**Corresponding author. Tel: +81-3-5463-0532, Fax: +81-3-5463-0532, E-mai l: urano(a)kaiyodai.ac.jp*

TABLE 1. Strains used in this study

Strain	Sampling site	PCR	LAMP
Klebsiella pneumoniae A12	Grit chamber (sewage treatment plant)	bla_{SHV}	+
Klebsiella pneumoniae B8	Discharged water (sewage treatment plant)	$bla_{CTX\text{-}M\text{-}9\ group}$	—
Escherichia coli D18	Reaction tank (sewage treatment plant)	$bla_{CTX\text{-}M\text{-}1\ group}$, bla_{TEM}	—
Escherichia coli hfa7	Tama River, Tokyo	$bla_{CTX\text{-}M\text{-}1\ group}$	—

tion of bla_{OXA} (Garciglia-Mercado et al., 2020), bla_{GES} (Takano et al., 2019) and $bla_{CTX\text{-}M}$ (Rivoarilala et al., 2018) genes of ESBL by the LAMP method. In the present study, we focused on developing a LAMP method for the rapid detection of bla_{SHV} gene, and we compared the sensitivity of the LAMP method and the conventional polymerase chain reaction (PCR) method. We also conducted a test of the direct detection of bla_{SHV} gene from water samples of the Tama River, which flows through metropolitan Tokyo on the borderline between Tokyo and Kanagawa prefectures.

MATERIALS AND METHODS

ESBL-producing bacterial strains

For the detection by LAMP method, we selected four strains (*K. pneumoniae* A12, *K. pneumoniae* B8, *E. coli* D18, and *E. coli* hfa7) from among the strains we obtained in previous studies. *K. pneumoniae* A12, *K. pneumoniae* B8, and *E. coli* D18 were isolated from a sewage treatment plant in Tokyo (Urano et al., 2020), and *E. coli* hfa7 was from the midstream bottom of the Tama River, Tokyo (Okai et al., 2019). The four strains were confirmed to be ESBL-producing strains by an antibiotic susceptibility test, a double disc synergy test (DDST), and a PCR assay (Table 1).

LAMP primer design

The bla_{SHV} primers were designed using Primer Explorer V5 software (https://primerexplorer.jp/lampv5/), based on the sequences of 44 bla_{SHV} genes (Table 2) from the GenBank database of the U.S. National Center for Biotechnology Information (NCBI). The primers set consisted of outer primers (F3 and B3), inner primers (FIP and BIP), and a loop primer (LF) (Fig. 1, Table 3).

Sensitivity of bla_{SHV} gene detection by the LAMP and conventional PCR assays

Each colony of the four strains was suspended in 100 µl of normal saline, and the 10^8-fold dilutions were spread on Luria-Bertani (LB) agar plates for the colony count. Total DNA was extracted by mixing 10 µl of each sample with 90 µl of Cica Geneus® DNA extraction reagent. Based on the colony count, we prepared the templates ranging from 10^6 to 1 DNA copy number in reaction tubes. The sensitivity was compared by three methods: LAMP without the LF loop primer, LAMP with the LF loop primer, and conventional PCR.

Each LAMP reaction was performed in a final volume of 25 µl containing each DNA template, 12.5 µl of 2× Reaction Mix (Eiken Chemical Co., Tokyo), 8 U of *Bst* DNA Polymerase (Eiken Chemical Co.), 0.2 µM F3 and B3 outer primers, 1.6 µM FIP and BIP inner primers, and 0.8 µM LF loop primer. Each tube was incubated at 65℃ for 1 hr and then heated at 80℃ for 5 min in a

TABLE 2. bla_{SHV} genes used for the design of LAMP primers

Protein name	Genbank ID	Protein name	Genbank ID
SHV-2	AF148851	SHV-57	AY223863
SHV-2a	DQ889460	SHV-64	DQ174304
SHV-3	KX092356	SHV-65	DQ174305
SHV-5	EF653399	SHV-66	DQ174306
SHV-7	AXLK01000004	SHV-67	DQ174307
SHV-8	U92041	SHV-69	DQ174308
SHV-12	AY259163	SHV-70	DQ013287
SHV-13	AF164577	SHV-86	DQ328802
SHV-15	AJ011428	SHV-98	AM922304
SHV-16	AF072684	SHV-99	AM922305
SHV-18	EU684753	SHV-102	EU024485
SHV-24	AB023477	SHV-104	EU274581
SHV-27	AF293345	SHV-105	FJ194944
SHV-30	AY661885	SHV-106	AM941847
SHV-31	AY277255	SHV-110	HQ877614
SHV-34	AY036620	SHV-115	MN550984
SHV-40	AF535128	SHV-128	GU932590
SHV-41	AF535129	SHV-129	GU827715
SHV-42	AF535130	SHV-134	HM559945
SHV-45	AF547625	SHV-145	JX013655
SHV-46	AY210887	SHV-148	JX121115
SHV-55	DQ054528	SHV-183	HG934764

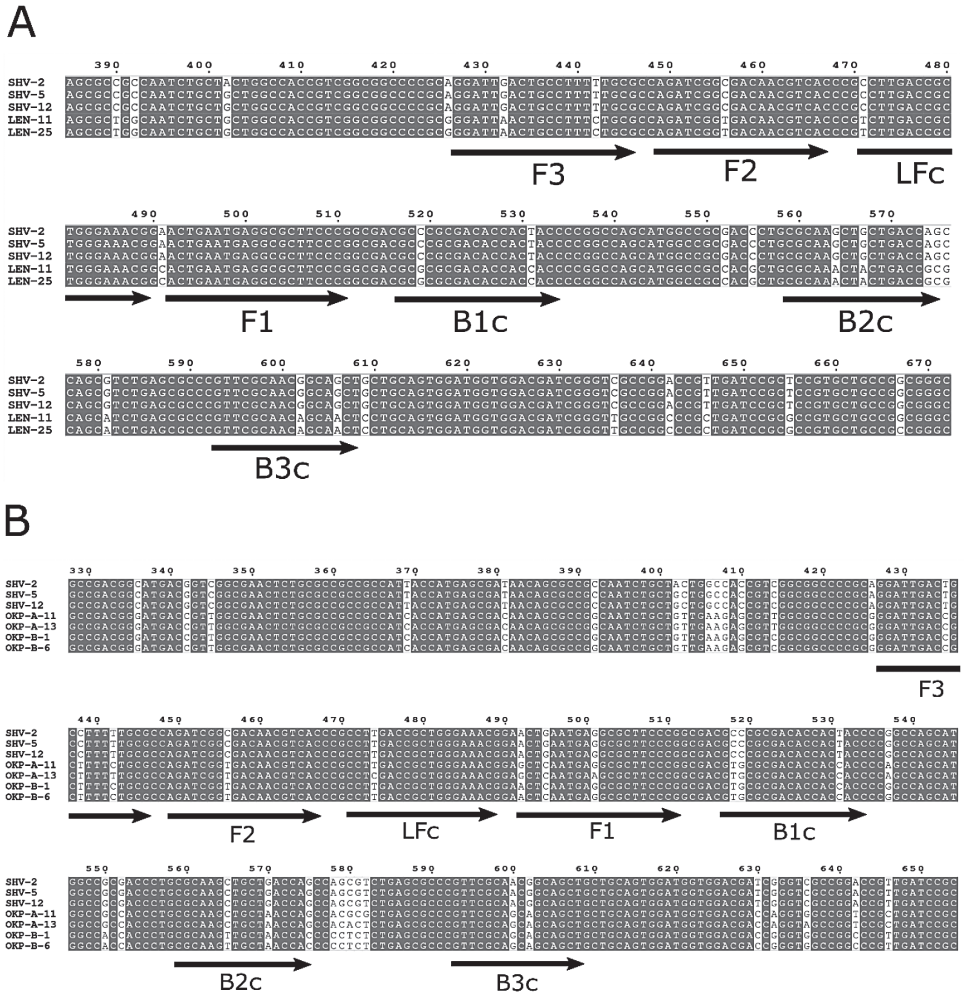

FIG. 1. Multiple sequence alignment of SHV, LEN, and OKP β-lactamase genes. (A) Sequence alignment of SHV (SHV-2 [Genbank: AF148851], SHV-5 [Genbank: EF653399], and SHV-12 [Genbank: AY259163]) and LEN (LEN-11 [Genbank: FLES01000010] and LEN-25 [Genbank: HQ709169]) β-lactamase genes. (B) Sequence alignment of SHV and OKP (OKP-A-11 [Genbank: AM850915], OKP-A-13 [Genbank: FJ534513], OKP-B-1 [Genbank: AJ635402], and OKP-B-6 [Genbank: AY850171]) β-lactamase genes. FIP primer consists of F1c and F2 sequences. BIP primer is composed of B1c and B2 sequences. The complementary sequences of F3, F2, F1, B1, B2, B3, and LF are F3c, F2c, F1c, B1c, B2c, B3c, and LFc, respectively.

TABLE 3. LAMP and sequence primers used in this study

Primer	Nucleotide sequence (5′ to 3′)
F3	GGATTGACTGCCTTTTTGCG
B3	AGCTGCCGTTGCGAAC
FIP (F1c-F2)	CGGGAAGCGCCTCATTCAGTAGATCGGCGACAACGTCAC
BIP (B1c-B2)	GCCCGCGACACCACTACCCTGGTCAGCAGCTTGCG
LF	CGTTTCCCAGCGGTCAAGG
F2	AGATCGGCGACAACGTCACGC
B2	CTGGTCAGCAGCTTGCGGC

Loopamp realtime RT-160C turbidimeter (Eiken Chemical Co.).

The PCR reaction volume was 20 µl containing each DNA template, 4.0 µl of reagent A (AptaTaq DNA Master), 7.0 µl of reagent B (PCR supplement), and 4.0 µl of reagent D (a mixture of $bla_{\text{CTX-M-25 group}}$, $bla_{\text{CTX-M chimera}}$, $bla_{\text{CTX-M-1 group}}$, and $bla_{\text{CTX-M-8 group}}$ primers or a mixture of bla_{SHV}, $bla_{\text{CTX-M-2 group}}$, $bla_{\text{CTX-M-9 group}}$, bla_{GES}, and bla_{TEM} primers) in the Cica Geneus ESBL Genotype Detection Kit2 (Kanto Chemical, Tokyo). The PCR reaction was performed in a TaKaRa PCR Thermal Cycler Dice system with an initial denaturation of 95℃ for 5 min, followed by 30 cycles of denaturation at 94℃ for 15 sec, annealing at 63℃ for 15 sec, and extension at 72℃ for 40 sec. The final extension step was carried out at 72℃ for 7 min.

The amplification products were analyzed by 2% agarose gel with Gel Green and visualized using an ImageQuant LAS 500 imager (Cytiva, Marlborough, MA, USA).

LAMP detection of bla_{SHV} gene from the Tama River water samples

River water samples were collected in October and December 2021 from seven sites (6, 13, 26, 37, 44, 61 and 81 km from the estuary) of the Tama River and transported to the laboratory in sterile bottles in contact with ice. For the removal of components in river water, the water sample that showed a positive result by LAMP assay was passed through a 0.22 µm membrane filter (Advantec, Tokyo), and microorganisms trapped on the membrane were suspended in a 1/100 volume of distilled water compared to the original river water volume. The suspension was serially ten-fold diluted. The sequencing of the amplification by LAMP was done with two sequence primers (F2 and B2) (Table 3) by Eurofins Genomics (Tokyo). The sequences were subjected to a BLAST analysis of the NCBI database.

RESULTS

Detection of bla_{SHV} gene by LAMP and conventional PCR

We confirmed the ESBL types of the four strains (*K. pneumoniae* A12, *K. pneumoniae* B8, *E. coli* D18, and *E. coli* hfa7) isolated in previous studies by conducting a conventional PCR using the Cica Geneus ESBL Genotype Detection Kit2. The ESBL types were as follows: *K. pneumoniae* A12, bla_{SHV}; *K. pneumoniae* B8, $bla_{\text{CTX-M-9 group}}$; *E. coli* D18, $bla_{\text{CTX-M-1 group}}$ and bla_{TEM}; *E. coli* hfa7, $bla_{\text{CTX-M-1 group}}$ (Table 1). The four strains were also investigated for the detection of bla_{SHV} gene by the LAMP method, and an increase of turbidity was observed in only *K. pneumoniae* A12 (Table 1), consistent with the results of the PCR assay.

Sensitivity of bla_{SHV} gene detection

We compared the sensitivity of LAMP and that of conventional PCR for the detection of bla_{SHV} gene from *K. pneumoniae* A12 by using seven reaction tubes that contained DNA templates ranging from 10^6 to 1 copy number. The detection limit without the LF loop primer was 10^5 copies/tube for the turbidity shown by the Loopamp realtime RT-160C turbidimeter. The results of agarose gel electrophoresis revealed clear bands in the range of 10^4 to 10^6 copies/tube (Fig. 2A). The detection limit of the LAMP method with the LF loop primer was 1 copy/tube (Fig. 2B), which is 10,000-fold more sensitive than the conventional PCR (Fig. 2C). The results are summarized in Table 4.

LAMP detection of bla_{SHV} gene from the Tama River water samples

To evaluate whether the bla_{SHV} gene from river water can be detected directly, we collected seven river water samples from the Tama River. As shown in Figure 2D, the LAMP assay confirmed the amplification of bla_{SHV} gene from one river water sample (26 km from the estuary). The sequence was 100% identical with a partial bla_{SHV} gene sequence (85 bp) from *K. pneumoniae* MS14393. We were unable to detect the amplification of bla_{SHV} gene by conventional PCR (Fig. 2E). Although we prepared the suspension containing 100-fold concentrated microorganisms without components in the river water, no bands were detected by the PCR assay (Fig. 2E).

DISCUSSION

The results of this study demonstrated that it is possible to detect the bla_{SHV} gene by a simple LAMP method. The LAMP assays were developed and optimized with five primers, and the detection limit with the loop primer was 10^4 to 10^5-fold more sensitive than that without the loop primer. In addition, the time that was necessary for the detection with the loop primer was approx. 20-30 min shorter than that without the loop primer. Thus, the loop primer plays an important role in the improvement of the detection limit and time by the LAMP method. The LAMP with the LF loop primer was 10^4-fold more sensitive than the conventional PCR. Differences in the sensitivity of LAMP and PCR were reported to range from 1- to 10,000-fold (Garciglia-Mercado et al., 2020; Rivoarilala et al., 2018; Moreira et al., 2018; Thirapanmethee et al., 2014). The LAMP method is thus a powerful tool for rapid and on-site detection of ESBL gene in environmental water sample.

Thirty four out of the 44 bla_{SHV} genes (Table 2) used

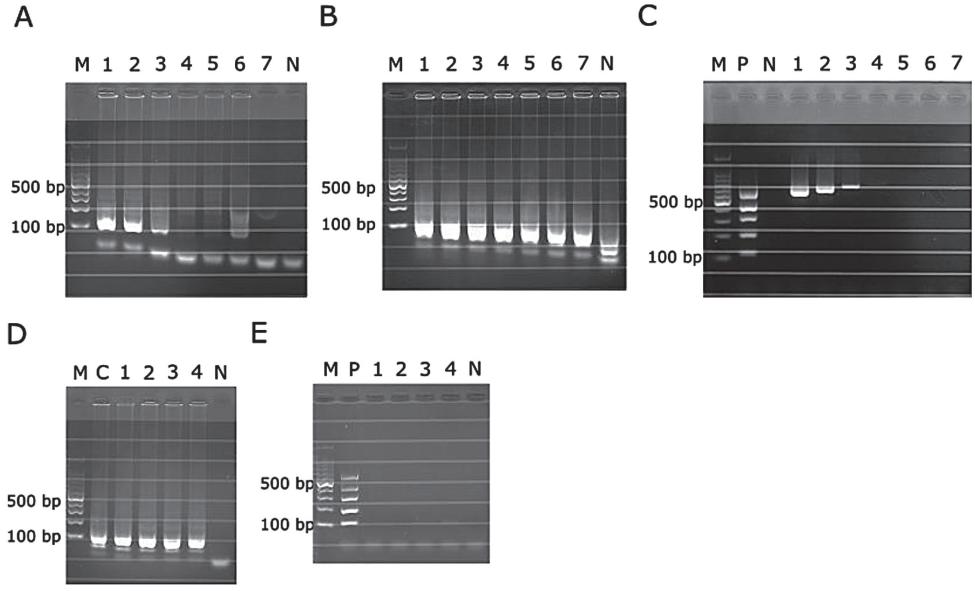

FIG. 2. Comparison of the utility of the LAMP and PCR assays for the detection of *bla*$_{SHV}$ gene from *K. pneumoniae* A12. The sensitivity of the LAMP assay was tested at 65℃ without the loop primer (A) and with the LF loop primer (B). The sensitivity of the PCR assay was determined using the Cica Geneus ESBL Genotype Detection Kit2 (C). The detection of *bla*$_{SHV}$ gene from the Tama River water sample (26 km from the estuary) was conducted by LAMP (D) and PCR (E) assays. Lane M: 100-bp DNA ladder marker. Lane P: positive control containing *bla*$_{SHV}$ (655 bp), *bla*$_{CTX\text{-}M\text{-}2}$ group (475 bp), *bla*$_{CTX\text{-}M\text{-}9\ group}$ (350 bp), *bla*$_{GES}$ (228 bp), and *bla*$_{TEM}$ (132 bp) genes in Cica Geneus ESBL Genotype Detection Kit2. Lane N: negative control containing distilled water. Lanes 1-7 of panels (A), (B) and (C): 10^6, 10^5, 10^4, 10^3, 10^2, 10, and 1 DNA copy numbers per reaction, respectively. Lanes 1 of panels (D) and (E): the original Tama River water sample. Lanes 2-4 of panels (D) and (E): the 100-, 10-, and 1- concentrated microorganism suspensions compared to the volume of the Tama River water sample. Lane C: control containing DNA template from *K. pneumoniae* A12.

TABLE 4. Agarose gel electrophoresis and turbidity of a Loopamp realtime turbidimeter for the LAMP and PCR assays

Copy number (CFU/reaction)	LAMP without LF		LAMP with LF		PCR
	Band in agarose gel	Turbidity	Band in agarose gel	Turbidity	
10^6	+	+	+	+	+
10^5	+	+	+	+	+
10^4	+	−	+	+	+
10^3	−	−	+	+	−
10^2	−	−	+	+	−
10	−	−	+	+	−
1	−	−	+	+	−

in this study had the same nucleotide sequences as the five LAMP primers, and ten (*bla*$_{SHV\text{-}3}$, *bla*$_{SHV\text{-}8}$, *bla*$_{SHV\text{-}24}$, *bla*$_{SHV\text{-}27}$, *bla*$_{SHV\text{-}445}$, *bla*$_{SHV\text{-}46}$, *bla*$_{SHV\text{-}70}$, *bla*$_{SHV\text{-}102}$, *bla*$_{SHV\text{-}105}$, and *bla*$_{SHV\text{-}110}$) had one nucleotide difference. Although it is difficult to estimate false-negative rate due to the number of samples, the LAMP primers seem to have sufficient specificity for the detection of *bla*$_{SHV}$ genes. In the phylogenetic tree of the ESBL genes (Fig. 3), the TEM and SHV families were clustered and related to each other, However, the nucleotide sequence identity in the LAMP primer regions was not high. Hæggman and co-workers revealed that the phylogenetic tree of

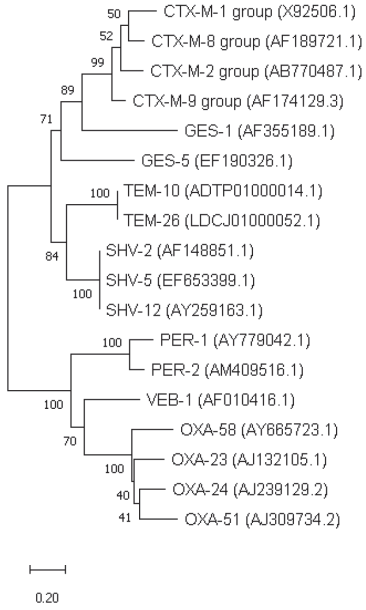

FIG. 3. Phylogenetic tree of ESBL genes. The phylogenetic tree was constructed in MEGA11 using the maximum likelihood method with a 1000 replicate bootstrap resampling.

SHV, LEN, and OKP β-lactamase genes showed parallel evolution, corresponding to the phylogenetic groups KpI, KpII, and KpIII, respectively (Hæggman et al., 2004). The five LAMP primers sequence showed high similarity (90-91%) with the corresponding regions of LEN and OKP β-lactamase genes (Fig. 1). Thus, it might be necessary to investigate false-positive by LAMP assay using LEN and OKP β-lactamase genes.

Our present results also confirmed the gene detection by the turbidity shown by a Loopamp realtime turbidimeter and agarose gel electrophoresis. Srisrattakarn et al. have developed a LAMP method with a stable, cost-effective, and easily visualized hydroxynaphthol blue (HNB) dye (Srisrattakarn et al., 2017). A further development of this and similar dyes would be necessary for on-site applications.

SHV-type ESBLs along with Temoneira (TEM)-type ESBLs were identified throughout the USA and Europe in the late 1980s and 1990s. They did not undergo an explosive dissemination, and the worldwide dominance of the CTX-M type has occurred (Doi et al., 2017). In the SENTRY program, most of the clinical isolates (*E. coli* and *K. pneumoniae*) carrying an ESBL gene harbored a CTX-M-type gene in the USA (92.5%), Europe (95.1%), the Asia-Pacific region (85.2%) and Latin America (98.1%) (Castanheira et al., 2021). The proportion of the SHV type in the USA was 8.6%. However, ESBLs have also been reported from a variety of environments other than hospital settings, food products, and livestock, and the SHV type is predominant depending on the samples. Romyasamit et al. reported that ESBL-producing *E. coli* and *K. pneumoniae* in Southern Thailand were present in raw vegetables at local markets, and the most frequently detected gene among them was bla_{SHV} (Romyasamit et al., 2021). In South Africa, the real-time PCR results of *Pseudomonas aeruginosa* isolates from abattoir wastewater (nonclinical samples) showed that 93.3% of the isolates harbored bla_{SHV}, 40% harbored bla_{TEM}, and 20% harbored bla_{CTX-M} (Hosu et al., 2021).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

Barthélémy, M., Péduzzi, J., Ben Yaghlane, H. and Labia, R. (1988) Single amino acid substitution between SHV-1 β-lactamase and cefotaxime-hydrolyzing SHV-2 enzyme. *FEBS Lett*., **231**, 217-220.

Bradford, P. A. (2001) Extended-spectrum β-lactamases in the 21st century: characterization, epidemiology, and detection of this important resistance threat. *Clin. Microbiol. Rev*., **14**, 933-951.

Castanheira, M., Simner, P. J. and Bradford, P. A. (2021) Extended-spectrum β-lactamases: an update on their characteristics, epidemiology and detection. *JAC-Antimicrobial Resist*., **3**, dlab092.

Chen, Z., Niu, H., Chen, G., Li, M., Li, M. and Zhou, Y. (2015) Prevalence of ESBLs-producing *Pseudomonas aeruginosa* isolates from different wards in a Chinese teaching hospital. *Int. J. Clin. Exp. Med*., **8**, 19400-19405.

Doi, Y., Iovleva, A. and Bonomo, R. A. (2017) The ecology of extended-spectrum β-lactamases (ESBLs) in the developed world. *J. Travel Med*., **24**, S44-S51.

Garciglia-Mercado, C., Gaxiola-Robles, R., Ascencio, F., Silva-Sánchez, J., Estrada-García, T. and Gómez-Anduro, G. (2020) Development of a LAMP method for detection of carbapenem-resistant *Acinetobacter baumannii* during a hospital outbreak. *J. Infect. Dev. Ctries*., **14**, 494-501.

Ghafourian, S., Sadeghifard, N., Soheili, S. and Sekawi, Z. (2015) Extended spectrum beta-lactamases: definition, classification and epidemiology. *Curr. Issues Mol. Biol*., **17**, 11-22.

Hæggman, S., Löfdahl, S., Paauw, A., Verhoef, J. and Brisse, S. (2004) Diversity and evolution of the class A chromosomal beta-lactamase gene in *Klebsiella pneumoniae*. *Antimicrob. Agents Chemother*., **48**, 2400-2408.

Hosu, M. C., Vasaikar, S., Okuthe, G. E. and Apalata, T. (2021) Molecular detection of antibiotic-resistant genes in *Pseudomonas aeruginosa* from nonclinical environment: public health implications in Mthatha, Eastern Cape Province, South Africa. *Int. J. Microbiol*., **2021**, 8861074.

Kliebe, C., Nies, B. A., Meyer, J. F., Tolxdorff-Neutzling, R. M. and Wiedemann, B. (1985) Evolution of plasmid-coded resistance to broad-spectrum cephalosporins. *Antimicrob. Agents Chemother*., **28**, 302-307.

Liakopoulos, A., Mevius, D. and Ceccarelli, D. (2016) A review of SHV extended-spectrum β-lactamases: Neglected yet ubiquitous. *Front. Microbiol*., **7**, 1374.

Moreira, M. G., Barreto, L. M., dos Santos, V. L., Monteiro, A.

S., Nobre, V. and dos Santos, S. G. (2018) Rapid detection of the New Delhi metallo-b-lactamase 1 (NDM-1) gene by loop-mediated isothermal amplification (LAMP). *J. Clin. Lab. Anal*., **32**, 1-5.

Okai, M., Aoki, H., Ishida, M. and Urano, N. (2019) Antibiotic-resistance of fecal coliforms at the bottom of the Tama River, Tokyo. *Biocontrol Sci*., **24**, 173-178.

Rivoarilala, O. L., Garin, B., Andriamahery, F. and Collard, J. M. (2018) Rapid in vitro detection of CTX-M groups 1, 2, 8, 9 resistance genes by LAMP assays. *PLoS One*., **13**, 1-15.

Romyasamit, C., Sornsenee, P., Chimplee, S., Yuwalaksanakun, S., Wongprot, D. and Saengsuwan, P. (2021) Prevalence and characterization of extended-spectrum-lactamase producing *Escherichia coli* and *Klebsiella pneumoniae* isolated from raw vegetables retailed in Southern Thailand. *PeerJ*., **9**, e11787.

Srisrattakarn, A., Lulitanond, A., Wilailuckana, C., Charoensri, N., Wonglakorn, L., Saenjamla, P., Chaimanee, P., Daduang, J. and Chanawong, A. (2017) Rapid and simple identification of carbapenemase genes, bla_{NDM}, bla_{OXA-48}, bla_{VIM}, bla_{IMP-14} and bla_{KPC} groups, in gram-negative bacilli by in-house loop-mediated isothermal amplification with hydroxynaphthol blue dye. *World J. Microbiol. Biotechnol*., **33**, 1-10.

Takano, C., Seki, M., Kim, D. W., Gardner, H., McLaughlin, R. E., Kilgore, P. E., Kumasaka, K. and Hayakawa, S. (2019) Development of a novel loop-mediated isothermal amplification method to detect guiana extended-spectrum (GES) β-lactamase genes in *Pseudomonas aeruginosa*. *Front. Microbiol*., **10**, 1-7.

Thirapanmethee, K., Pothisamutyothin, K., Nathisuwan, S., Chomnawang, M. T. and Wiwat, C. (2014) Loop-mediated isothermal amplification assay targeting the bla_{CTX-M9} gene for detection of extended spectrum β-lactamase-producing *Escherichia coli* and *Klebsiella pneumoniae*. *Microbiol. Immunol*., **58**, 655-665.

Urano, N., Okai, M., Tashiro, Y., Takeuchi, A., Endo, R., Ishida, M. and Takashio, M. (2020) Behavior of antibiotic-resistant fecal coliforms in the stream of a sewage treatment plant in Tokyo. *Adv. Microbiol*., **10**, 318-330.