ՀԱՅԱՍՏԱՆԻ ԳԻՏՈՒԹՅՈՒՆՆԵՐԻ ԱԶԳԱՅԻՆ ԱԿԱԴԵՄԻԱ
НАЦИОНАЛЬНАЯ АКАДЕМИЯ НАУК АРМЕНИИ
NATIONAL ACADEMY OF SCIENCES OF ARMENIA
ДОКЛАДЫ ՋԵԿՈՒՅՑՆԵՐ REPORTS

Հատոր Том Volume 122 2022 № 4

BIOLOGY

УДК 577:579.25:579.842.14
DOI: 10.54503/0321-1339-2022.122.4-307

S. S. Hakobyan, A. M. Sedrakyan

Plasmid Profiling in Extended Spectrum β-Lactamase-Producing Human Isolates of Non-Typhoidal *Salmonella*

(Submitted by corresponding member of NAS RA R. M. Aroutiounian 9/XI 2022)

Keywords: *non-typhoidal Salmonella, extended spectrum β-lactamases, plasmids, transformation.*

Current antimicrobial (AM) therapy recommendations for salmonellosis include third-generation cephalosporins; however, the emergence and spread of extended spectrum β-lactamase (ESBL) producing non-typhoidal *Salmonella* (NTS) as the result of the extensive use of cephalosporins has become a worldwide issue with serious consequences [1]. Previously, we reported the high prevalence of ESBL-producer phenotype among *Salmonella enterica* subsp. *enterica* serovar Typhimurium (hereinafter referred to as *S.* Typhimurium) isolates collected from patients in Armenia between 1996 and 2016 [2]. Based on whole genome sequencing (WGS) data, we identified the presence of $bla_{\text{CTX-M-5}}$ gene in human *S.* Typhimurium ST328 isolates displaying ESBL-producer phenotype and suggested that this phenotype is associated with plasmid-encoded CTX-M-5 production [2]. The CTX-M-5 is a β-lactamase of $bla_{\text{CTX-M-2}}$ phylogroup, which preferentially hydrolyses cefotaxime over ceftazidime and which is efficiently inhibited by clavulanic acid, tazobactam, and sulbactam [3, 4]. It was shown that $bla_{\text{CTX-M-5}}$ gene is predominantly located on plasmids, small non-self-transferable or conjugative [3, 5, 6]. In our previous work, we demonstrated the high prevalence of *in silico* predicted plasmids (pCTXM5-1358 and pCTXM5-637) in $bla_{\text{CTX-M-5}}$-positive human *S.* Typhimurium ST328 isolates from Armenia [2]. Of note, these mobilizable plasmids have been associated with the clonal distribution of multidrug-resistant *S.* Typhimurium ST328 isolates in several FSU countries [6]. Our previous work established a high degree of clonality among the human ESBL-producing *S.* Typhimurium isolates that have been circulating in Armenia for two decades [7]. In this study we explored the plasmid profile of human ESBL-producing *S.* Typhimurium isolates, presence of plasmid-encoded ESBL genes of $bla_{\text{CTX-M-2}}$

phylogroup in these isolates, as well as assessed the frequency of plasmid-mediated transfer of ESBL-producer phenotype.

Materials and methods. A total of 42 MDR *S.* Typhimurium isolates recovered from patients with salmonellosis over the period from 1996 to 2019 at the "Nork" Clinical Hospital of Infectious Diseases (MH) were included in this work. Of these, 37 isolates were collected in 1996-2014, 3 isolates were isolated in 2017, and 2 isolates – in 2019.

Determination of ESBL-producing phenotype. The ESBL-positive phenotype was identified by the double-disk synergy test using disks with ceftriaxone, ceftazidime, and amoxicillin-clavulanic acid according to the guidelines of the CLSI [8]. The results were verified using E-test strips (ESBL CT/CTL; BIOMÉRIEUX) according to the manufacturer's instructions. *E. coli* strains ATCC 25922 and ATCC 35218 were used for quality control.

DNA extraction. Total bacterial DNA samples for PCR analysis were isolated using the boiling lysate protocol [9]. Plasmid DNA was extracted using the E.Z.N.A.® Plasmid DNA Mini Kit I (OMEGA Bio-tech, Inc., USA) according to the manufacturer's recommendations. Plasmid DNA concentration was determined using a Micro Spectrophotometer (NanoDrop® ND-1000). DNA samples were stored at -20°C.

PCR-typing. Genes encoding β-lactamases of CTX-M family and CTX-M-2 phylogroup were detected by PCR as described previously [10, 11]. The following primers were used: i) 5'-ATGTGCAGYACCAGTAARGTKATGGC-3' and 5'-TGGGTRAARTARGTSACCAGAAYCAGCGG-3' for CTX-M family genes; ii) 5'-ATGATGACTCAGAGCATTCG-3' and 5'-GAAACCGTGGGTTACGATTT-3' for CTX-M-2 phylogroup genes. Detection of pCTX-M-5-like plasmids was performed as described previously [6] using the following primers: pCTXM5-F (5'-GACACAGAGAAGTTGATAGGCG-3') and pCTXM5-R (5'-TTCCAAATAGATCCACGGTGAC-3'). The amplified products were analysed by gel electrophoresis in 1.5% agarose. The GenScript PCR DNA Ladder M1060 was used as a molecular weight marker.

Plasmid DNA restriction. Plasmid DNA samples were digested with PstI and BglII endonucleases (Thermo Fisher Scientific), according to the manufacturer's recommendations. The digest products were separated by performing gel electrophoresis in 1% agarose. The 1 KB DNA Ladder (GenScript) was used as a molecular weight marker.

Plasmid-mediated transformation. Preparation of competent cells by $CaCl_2$ method was done as described previously [12]. Plasmids extracted from donor ESBL-producing *S.* Typhimurium isolates were transferred by transformation into the competent recipient isolates as described previously [13]. Transformed colonies were selected on agar plates containing ceftriaxone (4 mg/liter). Transformation efficiency was calculated as the number of colony forming units (cfu) per microgram of plasmid DNA used.

Results and discussion. A total of 42 human MDR *S.* Typhimurium isolates were tested for the phenotypic detection of ESBL-producers. Among these, 37 isolates from 1996-2014 were tested retrospectively to confirm the

presence of ESBL-producer phenotype identified earlier [2], while 5 isolates from 2017 and 2019 were tested for the first time. The results indicated that there was no ESBL-producing isolates among the clinical *S.* Typhimurium isolates from 2017 and 2019. Among the 37 isolates collected between 1996 and 2014, we detected 32 isolates showing ESBL-producer phenotype, while the remaining 5 isolates displayed ESBL-negative phenotype and were excluded from further analysis. It should be noted here that 18 out of 32 detected ESBL-producer isolates were subjected to WGS in our previous work [2].

PCR-detection of β-lactamase genes and pCTX-M-5-like plasmids. All isolates showing ESBL-producer phenotype (N=32) were tested for the presence of ESBL genes of $bla_{\text{CTX-M}}$ family and $bla_{\text{CTX-M2}}$ phylogroup. The results indicated that all ESBL-producer isolates in this study were positive for the genes mentioned (Table 1). The ESBL-producer isolates were also studied for the detection of pCTX-M-5-like plasmids. The presence of pCTXM5-like backbones was identified in 87.5% (28/32) of isolates (Table 1).

Plasmid profiles in ESBL-producing isolates. Plasmid DNA samples of 32 isolates were extracted and analysed by conventional agarose gel electrophoresis. The results indicated that in the three ESBL-producer isolates, which were subjected to WGS, plasmids cannot be detected on agarose gels. Of note, plasmids were also not predicted in these isolates using WGS data. All other ESBL-producer isolates in this study, 90.63% (29 out of 32 isolates), were found to carry plasmids. The most common plasmid was ~7.7 kb plasmid, identified in 81.25% (26/32) of isolates (Table 1). This plasmid detected alone in 71.87% of isolates was the most common plasmid profile (No. 1) identified in ESBL-producer isolates. In 9.37% of isolates (3/32), the 7.7 kb plasmid was found in combination with the other plasmids ranging in size. In one isolate, the other combination of two small plasmids of the sizes 7.6 and ~15 kb was found. In addition, we detected 2 isolates carrying a 6.5 kb plasmid.

PCR-typing of the plasmid DNA samples extracted from 29 plasmid-carrying ESBL-producer isolates revealed $bla_{\text{CTXM-2}}$ group genes and pCTXM5-like backbones in 96.55% (28/29) and 93.1% (27/29) of the isolates, respectively (Table 1).

Restriction fragment length polymorphism (RFLP) analysis of plasmid samples revealed 3 types of restriction profiles that are shown in Fig. 1. The most common RFLP profile, type I (Fig. 1, lines 4-6), was observed for the isolates carrying ~7.7 kb plasmid. Interestingly, the profile of restriction fragments mentioned was also detected in one isolate harboring 7.7 kb plasmid in combination with 3.7 kb band. The type II profile of restriction fragments (Fig. 1, line 1) was detected in the two isolates carrying 6.5 kb plasmids (Table 1, isolates ID: 1214 and A_678). In addition, the type III restriction fragments profile represented by a single ~15 kb band (Fig. 1, line 3) was found in the two isolates, carrying 7.7 kb plasmid in combination with ~45 kb plasmid (Table 1, isolates ID: A_6004 and A_8011), as well as in one isolate harboring 7.6 kb and 15 kb plasmids in combination (Table 1, isolate ID A_69).

Table 1

Plasmid profiles, CTX-M genes, and pCTXM5-like backbone of 32 human isolates of ESBL-producing *S.* Typhimurium

Plasmid profile number	Number of isolates (%)	Isolate ID	Year*	PCR-typing results					Plasmids detected by:	
				Total DNA			Plasmid DNA		Gel electro-phore-sis	*In silico* analysis
				bla$_{CTX-M}$ genes	*bla*$_{CTXM-2}$ genes	pCTXM5	*bla*$_{CTXM-2}$ genes	pCTXM5	Mol weight	*bla*$_{CTX-M-5}$ gene carrying plasmids
1	23 (71.85)	A_645	2006	+	+	+	+	+	7.7 kb	pCTXM5-1358**
		A_115, A_126	2011	+	+	+	+	+	7.7 kb	pCTXM5-1358
		4216	2011	+	+	+	+	+	7.7 kb	NA***
		A_1328, A_3194, A_3406, A_5962	2012	+	+	+	+	+	7.7 kb	pCTXM5-1358
		1349, 2324, 2730, 3128, 5703, 5730	2012	+	+	+	+	+	7.7 kb	NA
		3109	2013	+	+	+	+	+	7.7 kb	NA
		A_3040, A_3175, A_3246, A_3725	2014	+	+	+	+	+	7.7 kb	pCTXM5-1358
		1320, 2503	2014	+	+	+	+	+	7.7 kb	NA
		8019	2012	+	+	+	-	+	7.7 kb	NA
		8130	2014	+	+	+	+	-	7.7 kb	NA
2	2 (6.25)	A_6004, A_8011	2013	+	+	+	+	+	7.7 kb, 45 kb	pCTXM5-1358
3	1 (3.12)	5084	2011	+	+	+	+	+	3.7 kb, 7.7 kb	NA
4	2 (6.25)	A_678	2011	+	+	+	+	+	6.5 kb	pCTXM5-637**
		1214	2013	+	+	+	+	+	6.5 kb	NA
5	1 (3.12)	A_69	2006	+	+	-	+	-	7.6 kb, 15 kb	pCTXM5-637
6	3 (9.37)	A_60, A_684	1996	+	+	-	-	-	Not detected	Not detected
		A_3017	2013	+	+	-	-	-	Not detected	Not detected

* Year of isolation; ** pCTXM5-1358, accession No.: JX017308; pCTXM5-637 accession No.: JX017306; *** NA, not analysed.

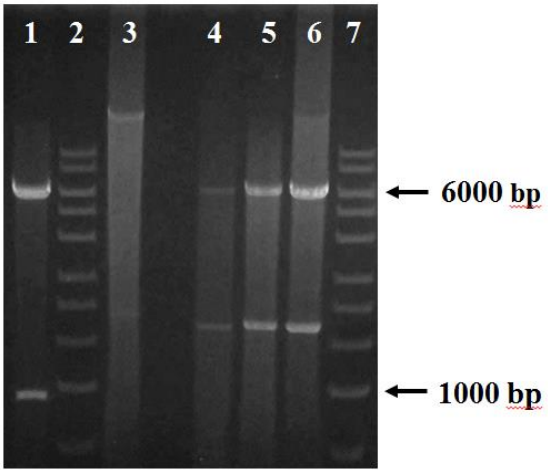

Fig. 1. Representative RFLP profiles of plasmids extracted from ESBL-producer *S.* Typhimurium isolates, which were obtained with PstI and BglII endonucleases. Lanes 4-6, type I profile; lane 1, type II profile; lane 3, type III profile; lanes 2 and 7, molecular weight marker: 1 KB DNA Ladder (GenScript).

It should be noted here that among 24 isolates showing the type I profile of restriction fragments, 11 isolates were subjected to WGS in our previous work [2]. *In silico* analysis revealed CTX-M-5-encoding plasmid pCTXM5-1358 (GenBank: JX017308) in all of these isolates [2]. The RFLP type I profile, consisting of the two bands with molecular weights of ~6 kb and ~1.7 kb, is in good agreement with the expected sizes of restriction fragments for pCTXM5-1358 plasmid (6055 bp and 1645 bp). The RFLP results as well as PCR-detected presence of $bla_{\text{CTXM-2}}$ group genes and pCTXM5-like backbones indicated that the remaining isolates, which demonstrated the same type I restriction fragments profile for 7.7 kb plasmid but were not subjected to WGS, also possessed the CTXM5-1358 plasmid encoding $bla_{\text{CTX-M-5}}$ gene. Plasmid content in one of the two isolates showing the type II RFLP profile (isolate ID A_678) was *in silico* analysed in our previous work [2] and CTX-M-5-encoding pCTXM5-637 plasmid (GenBank: JX017306) was predicted. Hence, it could be assumed that RFLP type II profile in the isolate, which was not sequenced (isolate ID: 1214) and was $bla_{\text{CTXM-2}}$ and pCTXM5-positive, is also associated with the prototype plasmid pCTXM5-637. Interestingly, we detected the same type III profile of restriction fragments in 2 isolates with *in silico* predicted pCTXM5-1358 plasmid (ID: A_6004 and A_8011) and in one isolate with *in silico* predicted pCTXM5-637 plasmid (ID: A_69).

Thus, the high prevalence of the CTX-M-5-encoding pCTXM5-1358 plasmids in human ESBL-producing *S.* Typhimurium isolates was revealed (81.25%), while the prevalence of the other CTX-M-5-encoding plasmid, pCTXM5-637, was lower (9.37%).

Plasmid transfer experiments. The efficiency of plasmid-mediated transfer of ESBL-producing phenotype was assessed using the plasmid DNA (7.7 kb plasmid, RFLP type I) of the two ESBL-producing *S.* Typhimurium isolates (ID: 1349 and A_3040) as donor isolates and competent *E. coli* DH5α strain, as well as the human NTS isolates belonging to the prevalent in Armenia serotypes of NTS isolates, which are susceptible to ceftriaxone, as recipients (Table 2).

Table 2

Transformation efficiency of different recipient strains by 7.7 kb plasmid.

Plasmid DNA donor	Recipient	Transformation efficiency (per 1 μg of plasmid DNA)
S. Typhimurium A_3040	*E. coli* DH5α	1.99×10^4 cfu/μg
	S. Typhimurium A_7687	7.17×10^1 cfu/μg
S. Typhimurium 1349	*S.* Enteritidis A_3972	4.01×10^3 cfu/μg
	S. Newport A_7187	6.83×10^2 cfu/μg

The ESBL-producing transformants were obtained from the both donor *S.* Typhimurium isolates that were used in these experiments. The results indicated that the transfer of ESBL-positive phenotype was observed not only to the competent *E. coli* DH5α strain, but also to the human *S.* Typhimurium, *S.* Enteritidis, and *S.* Newport isolates.

Thus, the high prevalence of the CTX-M-5 β-lactamase encoding 7.7 kb plasmids, sharing restriction profile and pCTXM5-like genetic backbone, was found in ESBL-producing *S.* Typhimurium isolates that were collected from patients in Armenia from 1996 to 2014. The results indicated that ESBL-producing phenotype of these isolates is generally associated with the carriage of pCTXM5-1358 plasmid, which can be transferred to other clinical isolates belonging to the common NTS serotypes in Armenia.

Institute of Molecular Biology of NAS RA
e-mail: a_sedrakyan@mb.sci.am

S. S. Hakobyan, A. M. Sedrakyan

Plasmid Profiling in Extended Spectrum β-Lactamase-Producing Human Isolates of Non-Typhoidal *Salmonella*

In this study we explored the plasmid profile of human extended spectrum β-lactamase (ESBL) producing non-typhoidal *Salmonella* (NTS) isolates, presence of plasmid-encoded ESBL genes of $bla_{\text{CTX-M-2}}$ phylogroup in these isolates, as well as plasmid-mediated transfer of ESBL-producer phenotype. The high prevalence of the 7.7 kb plasmids, encoding CTX-M-5 β-lactamase, as well as sharing restriction profile and pCTXM5-like genetic backbone, was found in ESBL-producing *S.* Typhimurium isolates. The results indicated that ESBL-producing phenotype of these isolates is

generally associated with the carriage of pCTXM5-1358 plasmids, which can be transferred to other clinical isolates belonging to the common in Armenia serotypes of NTS.

Շ. Ս. Հակոբյան, Ա. Մ. Սեդրակյան

Ընդլայնված սպեկտրի β-լակտամազներ արտադրող ոչ տիֆային *Salmonella* կլինիկական իզոլյատների պլազմիդային պրոֆիլները

Ուսումնասիրվել են ընդլայնված սպեկտրի β-լակտամազներ (ԸՍԲԼ) արտադրող ոչ տիֆային սալմոնելաների (ՈՏՍ) կլինիկական իզոլյատների պլազմիդային պրոֆիլները, պլազմիդներով կոդավորվող *bla*$_{\text{CTX-M-2}}$ ֆիլոխմբին պատկանող ԸՍԲԼ-ի գեների առկայությունը նշված իզոլյատներում, ինչպես նաև պլազմիդներով միջնորդավորված ԸՍԲԼ արտադրողի ֆենոտիպի փոխանցումը: ԸՍԲԼ արտադրող *S.* Typhimurium իզոլյատների մոտ հայտնաբերվել են ընդհանուր ռեստրիկցիոն պրոֆիլ և pCTXM5-տիպի գենետիկական հենք ունեցող CTX-M-5 β-լակտամազ կոդավորող 7.7 հազ.ն.զ. չափսով պլազմիդների բարձր տարածվածություն: Արդյունքները վկայում են, որ նշված իզոլյատների ԸՍԲԼ արտադրողի ֆենոտիպը հիմնականում կապակցված է pCTXM5-1358 պլազմիդների հետ, որոնք կարող են փոխանցվել Հայաստանում տարածված ՈՏՍ սերոտիպերին պատկանող այլ կլինիկական իզոլյատների:

Ш. С. Акобян, А. М. Седракян

Плазмидные профили выделенных от человека изолятов нетифоидных сальмонелл, продуцирующих β-лактамазы расширенного спектра

Исследованы плазмидные профили клинических изолятов нетифоидных сальмонелл (НТС), продуцирующих β-лактамазы расширенного спектра (БЛРС), наличие кодируемых плазмидами генов БЛРС филогруппы *bla*$_{\text{CTX-M-2}}$ в этих изолятах, а также опосредованная плазмидами передача фенотипа БЛРС-продуцента. Среди БЛРС-продуцирующих *S.* Typhimurium изолятов выявлена высокая распространенность кодирующих CTX-M-5 β-лактамазу плазмид размером 7.7 т.п.н., со сходным профилем рестрикции и pCTXM5-подобным генетическим каркасом. Результаты свидетельствуют о том, что фенотип продуцента БЛРС этих изолятов преимущественно ассоциирован с носительством плазмид pCTXM5-1358, которые могут передаваться другим клиническим изолятам, принадлежащим к распространенным в Армении серотипам НТС.

References

1. WHO (2017). Integrated Surveillance of Antimicrobial Resistance in Foodborne Bacteria: Application of a One Health Approach. Geneva: World Health Organization.
2. *Sedrakyan A. M., Ktsoyan Z. A., Arakelova K. A. et al.* – Front. Microbiol. 2020. 11. Article 592223. https://doi.org/10.3390/ijms23169330.

3. *Bradford, P. A., Yang, Y., Sahm, D. et al.* – Antimicrob. Agents Chemother. V. 42. P. 1980–1984.
4. *Tzouvelekis, L. S., Tzelepi, E., Tassios, P. T. et al.* – Int. J. Antimicrob. Agents. *V.* 14. P. 137–143.
5. *Edelstein, M., Pimkin, M., Dmitrachenko et al.* – Antimicrob. Agents Chemother. V. 48. P. 2808–2815.
6. *Kozyreva V. K., Ilina, E. N., Malakhova et al.* – Antimicrob. Agents Chemother. V. 58. P. 5202–5210.
7. *Sedrakyan A., Ktsoyan Z., Arakelova K. et al.* – Int. J. Mol. Sci. 2022. V. 23(16). 9330. https://doi.org/10.3390/ijms23169330.
8. CLSI. Performance Standards for Antimicrobial Susceptibility Testing, 30th ed.; CLSI Supplement M100; Clinical and Laboratory Standards Institute: Wayne, PA, USA, 2020.
9. *Pui C. F., Wong W. C., Chai L. C. et al.* – Food Control 2011. V. 22. P. 337–342.
10. *Hasman H., Mevius D., Veldman K. et al.* – J. Antimicrob. Chemother. V. 56. P. 115-121.
11. *Park Y. J., Lee S., Kim Y. R. et al.* – J. Antimicrob. Chemother. 2006. V. 57. P. 156-158.
12. *Xiaowei Li, Xin Sui et al.* – African J. of Biotechnology. 2010. V. 9 (50). P. 8549-8554.
13. *Hanahan D., Jessee J., Bloom F. R.* – Methods Enzymol. 1991. V. 20. P. 63-113.