Journal of Associated Medical Sciences 2024; 57(2): 86-100

JAMS
Journal of Associated Medical Sciences

Scopus Indexed & Thai-Journal Citation Index Centre (TCI)

Journal of Associated Medical Sciences

Journal homepage: https://www.tci-thaijo.org/index.php/bulletinAMS/index

CRISPR 2 spacer architecture analysis and virulotyping for epidemiological study of Salmonella enterica subsp. Enterica circulating in northern Thailand (2015 -2017)

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ARTICLE INFO

Article history:
Received 21 December 2023
Accepted as revised 13 February 2024
Available online 27 February 2024

Keywords:
CRISPR 2, spacer analysis, virulotyping, epidemiological study, rapid Salmonella typing, S. 4,[5],12:i:- subtyping.

ABSTRACT

Background: *Salmonella* enterica subsp. enterica, particularly serotype *S.* 4[5],12:i:-, *S.* Typhimurium, and *S.* Enteritidis, represents a significant causative agent of diarrhea, particularly impacting children and immunocompromised individuals on a global scale. Molecular typing of *Salmonella* spp. has a vital role in understand *Salmonella* epidemiology.

Objective: The objective of this study is to utilize CRISPR 2 spacer analysis coupled with multiple-locus variable number tandem-repeat (VNTR) analysis and virulotyping to perform molecular typing and potential subtyping of *Salmonella* spp.

Materials and methods: CRISPR 2 - multiple-locus variable number tandem-repeat (VNTR) analysis, complemented by additional virulotyping, were performed to rapidly characterize those *Salmonella* isolates including eight unidentified strains. Serotype-specific CRISPR 2 amplicons were subjected to sequencing and the obtained sequences were blasted with corresponding whole-genome sequencing (WGS) data in order to extract CRISPR 2 information, especially the number and sequence of spacers which were then utilized to predict *Salmonella* serotypes. Moreover, the similar CRISPR 2 spacer architectures to the corresponding WGS offered the prediction of multilocus sequence types (MLST).

Results: *S.* 4,[5],12:i:-, *S.* Typhimurium, *S.* Enteritidis, *S.* Weltevraden, and *S. Derby* exhibited distinct clustering, while eight unidentified *Salmonella* serotypes displayed unique CRISPR 2-MLVA profiles. Through subsequent sequence analysis and comparison with publicly available whole-genome sequencing data, serotype-specific CRISPR 2 amplicon lengths and spacer architectures were unveiled, enabling precise prediction of MLST types. Intriguingly, a linear correlation emerged between CRISPR 2 amplicon length (500-2000 bps) and the number of spacers (6-32) across diverse *Salmonella* serotypes. Critically, the molecular signatures of CRISPR 2 amplicons accurately predicted the identity of eight unknown *Salmonella* isolates, aligning with conventional serotyping standards. Furthermore, MLST sequences for prevalent *S.* 4,[5],12:i:-, *S.* Typhimurium, and *S.* Enteritidis were unveiled as ST 34, ST 19, and ST 10, respectively. Subtyping of *S.* 4,[5],12:i:- using the *sop*E1 procession (a bacteriophage gene) revealed two major subtypes within ST 34. These subtypes encompassed all six virulent genes, including *Inv*A, *bcf*C, *csg*A, *agf*A, *sod*C1, and *gip*A, either with *sop*E1 (N=8) or without *sop*E1 (N=10). These findings contribute preliminary insights into the genetic diversity and subtyping of *S.* 4,[5],12:i:-.

Conclusion: The combination of CRISPR 2 sequence analysis and virulotyping emerged as a potent epidemiological tool, facilitating the identification of *Salmonella* serotypes and potentially informative subtypes, thereby aiding in the surveillance, and tracking of *Salmonella* transmission in northern Thailand.

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doi: 10.12982/JAMS.2024.030
E-ISSN: 2539-6056

Introduction

Food poisoning or severe gastroenteritis resulting from the consumption of contaminated Salmonella spp. is a global issue.[1] Predominantly pathogenic to both warm-blooded animals and humans, S. enterica subsp. enterica can be classified into more than 2,463 serovars by assessing their antigenic properties according to the White-Kauffmann-Le Minor Scheme.[2] A limited collection of Salmonella serotypes potentially poses a public concern.[3] Non-Typhoidal Salmonella (NTS) is well-known for causing localized infections, diarrhea, or watery stools, particularly in young individuals.[4] Nevertheless, specific Salmonella serovars, such as S. Enteritidis and S. Typhimurium, are commonly associated with the development of more complicated sepsis.[5]

Nontyphoidal Salmonella (NTS) is commonly found in contaminated commercial meat, eggs, or dairy products. Common sources of the pathogen include well-known animal origins such as cattle, pigs, and chickens.[6] Transmission from animals to humans typically occurs through the consumption of contaminated food, often due to improper cooking or poor sanitation, particularly affecting vulnerable populations such as children, the elderly, or immunocompromised patients.[6,7]

Globally, the incidence of gastroenteritis is approximately 94 million cases per year, with documented rates ranging from 4 to 2741 cases per 100,000 people, varying geographically across the European Union and approximately 14 cases per 100,000 in the United States.[8,9] Furthermore, sporadic cases of multicountry outbreaks have been observed, such as those involving contaminated eggs with *S.* Enteritidis in Poland during 2016-2017 and powdered milk contaminated with *S.* Agona in France during 2017-2018.[10,11] In Thailand, the national documentation for the distribution of *Salmonella* serotypes was conducted during 1993-2002, involving more than 70,000 *Salmonella* isolates. The study identified groups of clinically important *Salmonella* serotypes, with *S.* Weltevreden ranking first, followed by *S.* Enteritidis (2nd), *S.* 4,5,12:i:- (4th), *S.* Typhimurium (5th), and *S.* Stanley (8th).[12] However, food poisoning was publicly documented at up to 163 cases per 100,000 in 2018, without specific causal characterization.[13]

Conventional typing, relying on serological characterization, is considered the standard method for *Salmonella* typing and is endorsed by the ISO system for official reporting.[14] However, the associated laboratory equipment is costly and skilled personnel are scarce. Advancements in *Salmonella* molecular typing have led to the development of rapid characterization methods, utilizing equipment ranging from standard PCR machines to advanced techniques like sequencing, NGS, and high-resolution melting temperature (HRM)-PCR, the latter often combined with multiplex PCR for detailed polymorphism detection.[15,19] For comprehensive epidemiological research, both conventional typing and appropriate molecular approaches are necessary to report *Salmonella* serovars and often their subtypes or even clonal identity in the case of outbreak analysis.[16] Molecular targeting of *Salmonella* typing is rapidly achieved by identifying specified polymorphic alleles of 16S RNA, the *rfb* gene cluster involved in O-antigen biosynthesis, and flagella genes.[17,18] More intricate protocols utilizing advanced technology have been developed to precisely characterize bacterial subtypes or identify clones. Various approaches, including Multilocus Sequence Typing (MLST), Multi Locus Variable number of tandem repeat Analysis (MLVA), Pulsed Field Gel Electrophoresis (PFGE), and Whole Genome Sequencing (WGS), have been established for epidemiologically identifying the transmission routes of *Salmonella* spp. or the causal origins of *Salmonella* outbreaks.[20,21]

Clustered regularly interspaced short palindromic repeats (CRISPR) constitute a novel family of repeated DNA sequences recognized for their adaptive immune protection against foreign mobile genetic elements (MGEs) present in various gram-negative bacteria, including *E.coli*, *Salmonella* spp., and *S.Klebsiella pneumoniae*.[22,23] The distinctive genetic features of the CRISPR locus include an alternating series between direct repeats (DRs), 24-47 bp in length, and DNA variable sequences (spacers) of 21-72 bp.[24] Positioned upstream to the CRISPR locus are a "leader sequence", alternated repeated patterned of directed sequences (DR) and specific spacers and followed by several types of (CRISPR-associated sequence) genes.[25] The CRISPR locus potentially houses unique genetic sequences influenced by evolutionary pressures and is commonly employed for bacterial typing or clonal identification. In *Salmonella* typing, previous studies compared sequence polymorphisms of two CRISPR loci, CRISPR 1 and CRISPR 2, across 783 *Salmonella* strains, observing correlations between *Salmonella* serotypes and multilocus sequence types.[26] Additionally, the characterization of spacer content within CRISPR alleles provided sufficient information for *Salmonella* subtyping.[27] In practical applications, a CRISPR 2 PCR assay was developed for the rapid characterization of three clinically important *Salmonella* spp. using agarose gel electrophoresis.[28] More recently, CRISPR loci polymorphisms have been utilized in a single-step assay to identify multiple contaminated *Salmonella* spp. from poultry samples.[29] As a molecular tool for subtyping bacterial pathogens, multiple-locus variable number tandem-repeat analysis (MLVA) relies on characterizing copy numbers of tandem repetitive DNA elements at different loci. The development of MLVA primarily involves selecting potential loci and designing multiplex primers.[30] This approach has been extensively applied to subtype *S.* Enteritidis and *S.* Typhimurium, demonstrating a high discriminatory index based on amplicon sizing from capillary electrophoresis.[31] Notably, recent MLVA methods have shown promise in characterizing *E. coli* subtypes corresponding to Sequence Types (ST) through visual observation on agarose gel electrophoresis.[32]

Virulotyping or detecting different profiles of virulent gene panels was employed as a potential tool to characterize *Salmonella* heterogenicity among different *Salmonella* serotypes or the same *Salmonella* serovars based on their different phenotypic characteristics. Virulotyping 523 *Salmonella* enterica Serovars Relevant to

Human Health in Europe showed the distribution of most virulence genes corresponding with *Salmonella* serotypes; nevertheless, high variation in the same serotypes was observed with those virulence genes that were prophage encoded, in fimbrial clusters or in the virulence plasmid.[33] The *Salmonella* subtyping of *Salmonella* Typhimurium by differentiating prophage profiles was further performed and found that prophage profiles significantly correspond to MLVA types.[34]

To assess molecular typing methods targeting CRISPR 2 loci, MLVA, and virulent genes as an alternative approach for *Salmonella* typing, preliminary analyses of CRISPR 2-MLVA and CRISPR 2 sequence were rapidly conducted. This evaluation aimed to differentiate 51 standard *Salmonella* strains from various sources (part of a standard collection) and eight *Salmonella* isolates of unknown origin. Moreover, the process involved the sequencing of CRISPR 2 amplicons to determine *Salmonella* serotypes and MLST sequences. These sequences were then compared to the *Salmonella* whole-genome sequencing (WGS) public database available on the public bacterial database (enterobase.com). Furthermore, additional virulent genes were selected and analyzed to provide preliminary information for characterizing *Salmonella* subtypes. This information could potentially offer insights into *Salmonella* transmission patterns in northern Thailand.

Materials and methods

Salmonella isolates in this study

This study analyzed 59 *Salmonella* isolates, comprising 17 identified serotypes via conventional methods and 8 unidentified strains (Table 1). The isolates included 43 samples from hospitalized patients, collected at Phayao Ram Hospital, Phayao Province, between 2015-2017.[35] The remaining 16 isolates were sourced from minced pork and various animal species, acquired across five northern Thailand provinces during 2017-2018.[36]

The collection and use of clinical *Salmonella* isolates were ethically approved by Phayao University's Ethical Committee (Approval No. 57 02 04 0020). Similarly, the procurement of animal-derived isolates adhered to ethical standards, with approval granted by the University's Institutional Animal Care and Use Committee (IACUC), under Project No. 62-02-04-001.

Table 1 Collection of 59 *Salmonella* isolates from various sources in northern Thailand (2015-2017).

Number	Serotypes	Source	Number (N=59)
1	*S.*4,5,12:i:-	Hospitalized patients	16
		Minced pork	4
		Swine	1
2	*S.*Typhimurium	Chicken	2
		Goat	1
3	*S.*Enteritidis	Hospitalized patient	5
		Chicken	1
4	*S.*Weltevreden	Hospitalized patient	4
5	*S.*Stanley	Hospitalized patient	3
6	*S.*Derby	Minced pork	2
7	*S.*Kentucky	Hospitalized patient	1
8	*S.*Agona	Chicken	1
		Hospitalized patient	1
9	*S.*Schwarzen	Minced pork	1
10	*S.*Covallis	Hospitalized patient	1
11	*S.*Krefeld	Swine	1
12	*S.*Braenderup	Chicken	1
13	*S.*Saintpaul	Minced pork	1
14	*S.*Montevideo	Hospitalized patient	1
15	*S.*Barilly	Hospitalized patient	1
16	*S.*Kedougou	Hospitalized patient	1
17	*S.*Give	Hospitalized patient	1
18	Unknown	Hospitalized patient	8

Molecular analyzation of Salmonella isolates from various sources was conducted by performing CRISPR 2 MLVA PCR

Salmonella strains stored in glycerol stocks at -20°C were revived and incubated in NB broth overnight. Confirmation of *Salmonella* identity was conducted by plating on MacConkey agar and SS agar, respectively. A single black colony was selected from SS agar for subsequent DNA isolation. *Salmonella* DNA extraction followed a previously described method.[39] In brief, a one mL aliquot of the culture underwent centrifugation, and the pellet was washed with TE buffer (10 mM Tris HCl, pH 8.0, containing 1 mM EDTA). Subsequently, it was suspended in 400 µL of TE buffer and heated at 80°C for 20 min. Then, 50 µL of lysozyme solution (10 mg/mL) was added, and the mixture was incubated at 37°C for one hr. Following this, 75 µL of a 10% SDS/proteinase K solution was added, and the mixture was incubated at 65°C for 10 min. Next, 100 µL aliquots of 5 M NaCl and prewarmed (65°C) cetyl trimethylammonium bromide (CTAB)/NaCl solution were added. The solution was then incubated at 65°C for 10 min, followed by the addition of 750 µL of chloroform:isoamyl alcohol (24:1) solution. The mixture underwent centrifugation at 11,000×g for 5 min at 4°C, followed by ethanol precipitation. Lastly, the DNA pellet was dissolved in 50 µL of distilled water and stored at -20°C until use.

Multiplex PCR was conducted using specific sets of primers for the amplification of CRISPR 2 alleles and MLVA, as detailed in Table 2. The PCR reactions were performed in a BIO-RAD CFX96TM Real-Time System (Bio-Rad, Hercules, CA, USA). Each PCR mixture (10 µL) contained 1 µL of DNA, 8 pairs of primers at concentrations specified in Table 1, and 2 µL of HOT FIREPol Blend Master Mix Plus 10 mM $MgCl_2$ (Solis Biodye, Tartu, Estonia). The thermocycling conditions for the MLVA set were as follows: initial denaturation at 95°C for 12 minutes, followed by 30 cycles of 94°C for 30 sec, 55°C for 90 sec, and 72°C for 90 sec, with a final extension step at 72°C for 10 minutes. For the CRISPR 2 PCR, the thermocycling conditions included an initial denaturation at 95°C for 12 min, followed by 35 cycles of 94°C for 60 sec, 59°C for 90 sec, and 72°C for 90 sec, with a final extension step at 72°C for 10 min. Subsequently, amplicons from both reactions were combined and subjected to 1% agarose gel electrophoresis in Tris Borate EDTA (TBE, 1X) buffer. Visualization was achieved by staining with RedSafe dye (1:20,000) (INiRON, Washington, United States).

Table 2. Primers used in this study.

Primer	Genes	Sequence (5' → 3')	Size of PCR- product (bps)	Primer Concentration (pmol/ul)	Reference
***Salmonella* typing**					
CHRISPR 2 and MLVA multiplex					
B1	CRSPR 2 loci	GAGCAATACYYTRATCGTTAACGCC	variable	0.2	[24]
B2		GTTGCDATAKGTYGRTRGRATGTRG		0.2	
ECMLV1_f		TCCCTGGACAAACCAGGACTG	162	0.1	[30]
ECMLV1_r1		CGTGCGGACTTATGAGAAAG		0.1	
ECMLV1_r2		CGTGCGGGCTTATGAAAAAG		0.5	
ECMLV2_f		GAAACAGGCCCAGGCTACAC	575	0.05	
ECMLV2_r		CTGGCGCTGGTTATGGGTAT		0.05	
ECMLV3_f		TTCAGGAAATGGATAAAGTAGT	616	0.8	
ECMLV3_r		GGGAGTATGCGGTCAAAAGC		0.8	
ECMLV4_f		ACAACCGGCTGGGGCGAATCC	413	0.05	
ECMLV4_r		GTCAGCAAATCCAGAGAAGGCA		0.05	
ECMLV5_f		GCGGCGCTGAAGAAGAAAGC	413	0.05	
ECMLV5_r		CTCCCGGCAGGCGAAGCATTGT		0.05	
Virulotyping					
Multiplex 1 bcfC; Bovine colonization factor, fimbrial usher, **csgA;** Major fimbrial subunit of thin curled fimbriae and **agfA;** Aggregative fimbria A					
bcfC_f		CAGCTTTTCATGACGCGATA	241	0.4	[35]
bcfC_r		CAATGTCTCTGGTTGCGAGA		0.4	
csgA_f		GGATTCCACGTTGAGCATTT	212	0.4	
csgA_r		CGGAGTTTTTAGCGTTCCAC		0.4	
agfA_f		GGATTCCACGTTGAGCATTT	312	0.4	
agfA_r		GTTGTTGCCAAAACCAACCT		0.4	
Uniplex 1 invA location; Chromosome, Function Enables the bacteria to invade cells					
InvA_f		GTGAAATTATCGCCACGTTCGGGCAA	284	0.125	[36]
InvA_r		GCCCCGGTAAACAGATGAGTATTGA			
Uniplex 2 sopE1 location; Cryptic bacteriophage, Function ; Translocated T3SS effector protein					
sopE1_f		CGGGCAGTGTTGACAAATAAAG	422	0.4	[31]
sopE1_r		TGTTGGAATTGCTGTGGAGCT		0.4	

Table 2. Primers used in this study. *(continued)*

Primer	Genes	Sequence (5' → 3')	Size of PCR- product (bps)	Primer Concentration (pmol/ul)	Reference
Uniplex 3 gipA l location; Gifsy- 1bacteriophage, Function; Peyer's patch-specific virulence factor					
gipA_f		ACGACTGAGCAGGCTGAG	518	0.4	[31]
gipA_r		TTGGAAATGGTGACGGTAGAC		0.4	
Uniplex 4 sodC1 location; Gifsy-2bacteriophage, Function; periplasmic Cu, Zn superoxide dismutase					
sodC1_f		CCAGTGGAGCAGGTTTATCG	424	0.4	[31]
sodC1_r		GGTGCGCTCATCAGTTGTTC		0.4	

[b]Y=T or C; R=A or G; S=G or C; D=A or G or T

Amplicon Profile Analysis and Phylogenetic Tree Construction.

CRISPR 2-MLVA amplicon patterns were analyzed using a curve-based algorithm (Pearson correlation) in the open-source GelJ software.[40] This approach allowed for the creation of a similarity scale, essential for comparing amplicon size variations among different *Salmonella* serotypes. The resultant amplicon profiles were then used to construct phylogenetic trees. An unweighted pair-group method using arithmetic averages (UPGMA) algorithm was employed for this purpose, chosen for its efficacy in illustrating the evolutionary relationships among closely related isolates.

Characterization of CRISPR 2 amplicon sequences

CRISPR 2 amplicons for spacer arrangement analysis were individually examined in separate uniplex experiments for thirty-three isolates (nos. 4, 38, 52, 125, 131, 132, 133, 145, 246, 254, 262, 378, 411, 413, 426, 443, 450, 454, 466, 475, 482, 483, 487, 495, L8, L10, L17, L27, en6, en13, en18, en8, en11). These amplicons were excised and sent for DNA sequencing at First Base (First BASE Laboratories Sdn Bhd, Malaysia).

The obtained CRISPR 2 sequences, in FASTA format, were analyzed using the CRISPRCasFinder tool (https://crisprcas.i2bc.parissaclay.fr/CrisprCasFinder/Index) set to default parameters. This tool was chosen for its accuracy in identifying CRISPR 2 features such as the DR consensus sequence, DR length, and spacer count. Data was presented as sequences or numerical values for further analysis.

Additionally, these CRISPR 2 sequences were compared with Whole Genome Sequences (WGS) of corresponding Salmonella serotypes from the public domain. BLAST analysis, using CRISPR 2 specific primers, was conducted to determine the congruence in amplicon lengths, as visualized in agarose gel electrophoresis.

Molecular analysis of* Salmonella virulent *genes

Amplifications of seven virulent genes were carried out using either multiplex or uniplex PCR with primers obtained from IDT, Singapore (refer to Table 2). Each reaction mixture (10 µL) comprised 1 µL of DNA, the primer set at concentrations specified in Table 2, and 2µL of HOT FIREPol Blend Master Mix Plus 10 mM $MgCl_2$ (Solis Biodye). For all multiplexes and uniplexes, the thermocycling conditions were as follows: an initial denaturation at 95°C for 15 min, followed by 35 cycles of 95°C for 30 sec, 58°C for 30 sec, and 72°C for 1 min, with a final extension step at 72°C for 5 min. The exception was uniplex PCR 1, where the annealing temperature was set at 64 °C for invA. Following amplification, the resulting amplicons were visualized through 1.5% agarose gel electrophoresis, stained with RedSafe dye (1:20000) (INiRON, Washington, United States).

Statistical analysis

Data analysis for descriptive statistics was performed by using of SPSS for Windows, version 10 (SPSS Inc, Chicago, USA) at the University of Phayao.

Results

CRISPR 2-MLVA Profiles in Clinically Important Salmonella Serotypes

The rapid molecular typing of 59 *Salmonella* isolates, including 17 identified and 8 unknown serotypes from northern Thailand (2015-2017), revealed distinct CRISPR 2-MLVA profiles (Figure 1). These profiles allowed us to categorize the isolates into three clusters. Specifically, S. 4,5,12:i:- (N=21), *S.* Enteritidis (N=6), and *S.* Derby (N=2) showed unique profiles with CRISPR 2 fragments at approximately 1600 bps, 800 bps, and 900 or 950 bps, respectively. In contrast, the third cluster exhibited closely related profiles among *S.* Typhimurium, *S.* Stanley, and *S.* Weltevreden, with variations in CRISPR 2 amplicon sizes (1700 bps for *S.* Typhimurium; 1600, 1650, and 1500 bps for *S.* Weltevreden; 1600 and 1500 bps for *S.* Stanley). The other nine serotypes and the eight unidentified isolates presented diverse CRISPR 2-MLVA patterns, with notable faint bands in *S.* Kreldfeld (L8) and *S.* Braenderup (L12), and similar profiles in *S.* Saintpaul (en18) and *S.* Montediveo (38) at approximately 1500 bps.

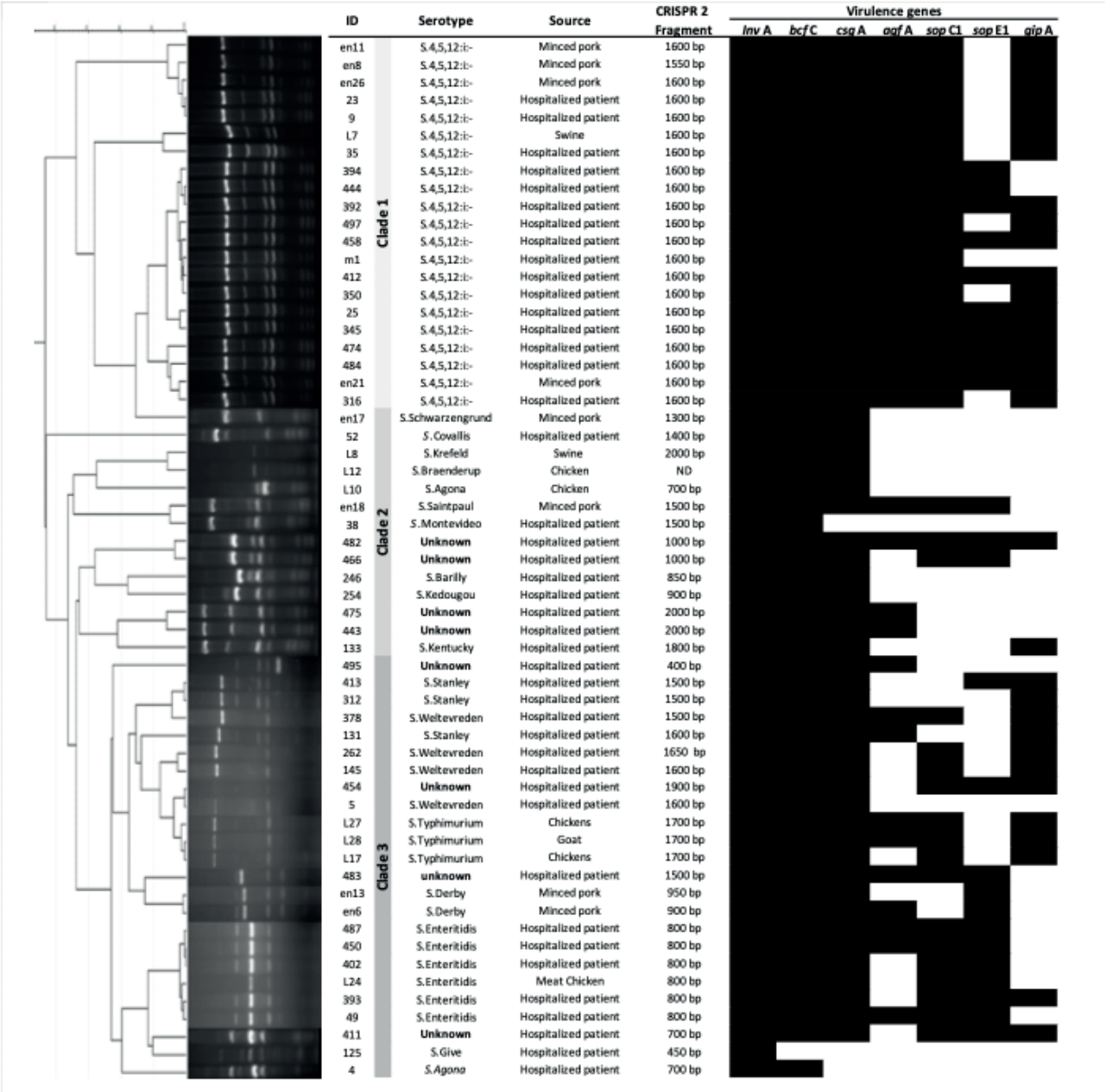

Figure 1 *CRISPR 2 - MLVA Phylogeny tree with associated virulotypes of 59 Salmonella isolates from various sources in northern Thailand (2015-2017). ID: abbreviated name of Salmonella isolates.*

In summary, the CRISPR 2-MLVA profiles of *S.* 4,5,12:i:-, *S.* Enteritidis, and *S.* Derby were consistently distinct and appear suitable for *Salmonella* typing. Further sequence analysis is planned to explore the CRISPR 2 amplicons in more detail, focusing on direct repeats, spacer numbers, and array structures, to confirm serotypes and characterize the unknown isolates.

CRISPR 2 amplicon sequence analysis of 17 *Salmonella* serotypes and 8 unknowns

The analysis of CRISPR 2 loci in 17 identified and 8 unknown *Salmonella* serotypes revealed amplicons ranging from 500 to 2500 bps. Notably, *S.* Braenderup did not produce any CRISPR 2 amplicons (Figure 2). Serotypes like *S.* Give, *S.* Agona, *S.* Enteritidis, *S.* Schwarzen, and *S.* Covallis displayed unique amplicon lengths (480 to 1300 bps). Others, including *S.* 4,5,12:i:- and *S.* Typhimurium, generated amplicons around 1500 bps, indicating serotype-specific profiles.

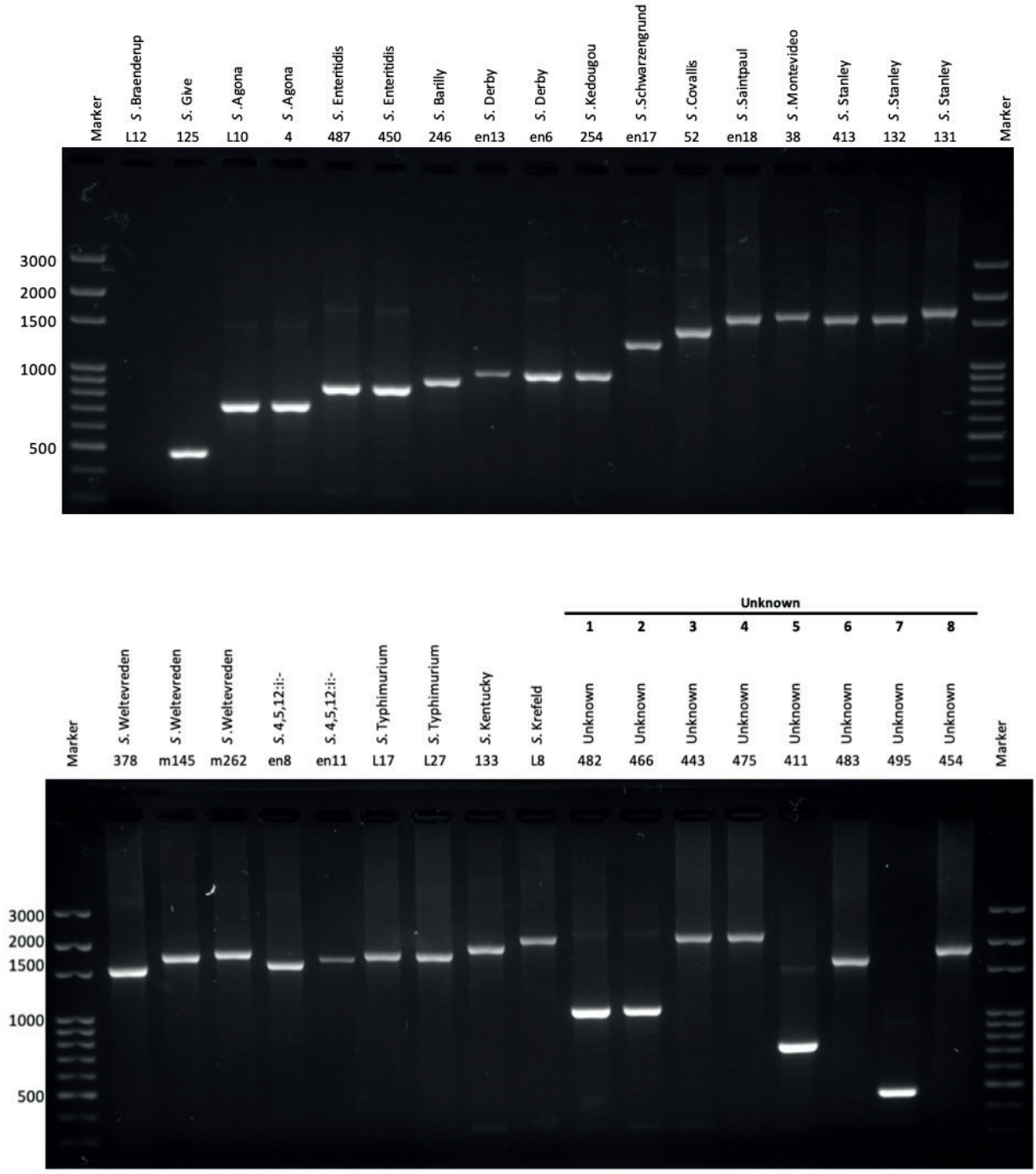

Figure 2 *The CRISPR 2 amplicons of 17 Salmonella serotypes and 8 unknowns of Salmonella isolates from various sources in northern Thailand (2015-2017).*

We sequenced CRISPR 2 amplicons from a representative sample (17 serotypes, 26 isolates; 8 unknowns, 8 isolates) to analyze spacer profiles. Comparing these with public WGS data, we found a close match in molecular weights and spacer counts (Table 3). This alignment suggests a strong correlation between CRISPR 2 amplicons and *Salmonella* serotypes. Particularly, *S.* 4,5,12:i:-, *S.* Typhimurium, and *S.* Enteritidis produced serotype-specific CRISPR 2 amplicons correlating with ST 34, ST 19, and ST 11, respectively. *S.* Stanley and *S.* Weltevreden had similar-sized amplicons corresponding to ST 29 and ST 365. The unknown isolates were identified as various serotypes, including *S.* Typhimurium and *S.* Weltevreden, with novel spacer additions predicted in some cases.

These findings demonstrate the practical utility of CRISPR 2 spacer analysis in predicting *Salmonella* serotypes and STs, offering a viable alternative to conventional typing methods. Furthermore, a linear relationship between the CRISPR 2 amplicon lengths and the number of spacers was observed (Figure 3), underscoring the structured molecular system and potential of using CRISPR 2 loci for indicating other *Salmonella* serotypes from this study.

Table 3 Description of some *Salmonella* serotype specific spacers extracted from raw sequences and further analyzation of the corresponding WGS reference of 17 *Salmonella* serotypes and eight unknowns from various sources in northern Thailand (2015-2017).

NO	Isolated	#C2 Fragment	$S1	S2	S3	S7	S8	S9	S16	S17	Serotypes	Expected C2 Fragments	Expected total number of spacers C2	Expected ST	WGS data Reference
1	L12	0									*S*.Braenderup	0	18	22	SAL_BA8466AA
2	m125	450	ParAB0	CheB1	CheB2						*S*.Give	465	6	516	SAL_XA8594AA
3	L10	700	AgoB1	AgoB2	AgoB3	AgoB7	AgoB8				*S*.Agona	752	8	13	SAL_DB2207AA
4	m4	700	AgoB1	AgoB2	AgoB3	AgoB7	AgoB8				*S*.Agona	752	8	13	SAL_DB2207AA
5	487	850	EntB0	EntB1	EntB2	EntB6	EntB7	EntB8			*S*.Enteritidis	833	10	11	SAL_XA8590AA
6	450	850	EntB0	EntB1	EntB2	EntB6	EntB7	EntB8			*S*.Enteritidis	833	10	11	SAL_XA8590AA
7	m246	900	STMB0	STMB32	STMB2	STMB32	STMB4	STMB5			*S*.Barilly	950	11	909	SAL_XA8606AA
8	en13	1000	DerB1	DerB2	DerB3	DerB13	DerB7	DerB9			*S*.Derby	1014	13	40	SAL_FA4928AA
9	en6	1000	DerB1	DerB2	DerB3	DerB13	DerB7	DerB9			*S*.Derby	1014	13	40	SAL_FA4928AA
10	m254	1000	EnuB1	EnuB2	3	7	Newp13	kra16var			*S*.Kedougou	953	12	1543	SAL_XA8603AA
11	en17	1300	ParBB5	STMB32	BovB1	Swa3	Swa4	Swa5	SwaB12	SwaB13	S. Schwarzengrund	1258	17	96	SAL_DA7383AA
12	m52	1400	MaraB1	NapB4	AltB23	LexB8	LexB8	9	SenB13	JavB1	S.Covallis	1441	20	1541	SAL_XA8588AA
13	en18	1500	ParBB1	StpB3	StpB27	StpB15	StpB7	StpB8	StpB22	StpB23	*S*.Saintpaul	1563	22	50	SAL_FA2386AA
14	m38	1550	JavB1	MonB39	MonB40	MonB44	MonB45	MonB56	MonB46	MonB48	*S*.Montevideo	1625	23	1531	SAL_XA8587AA
15	413	1500	EntB0	IndB1	EntB10	NewpB34var1	BloB1	MbaB38	NiaB2	StaB6	*S*.Stanley	1686	24	29	SAL_XA8593AA
16	132	1500	EntB0	IndB1	EntB10	NewpB34var1	BloB1	MbaB38	NiaB2	StaB6	*S*.Stanley	1686	24	29	SAL_XA8593AA
17	131	1600	EntB0	IndB1	EntB10	NewpB34var1	BloB1	MbaB38	NiaB2	StaB6	*S*.Stanley	1686	24	29	SAL_XA8593AA
18	378	1500	CholB16	WelB2	WelB3	WelB5	WelB6	WelB7	WelB22	WelB23	*S*.Weltevreden	1808	26	365	SAL_XA8578AA
19	m145	1770	CholB16	WelB2	WelB3	WelB5	WelB6	WelB7	WelB22	WelB23	*S*.Weltevreden	1808	26	365	SAL_XA8578AA
20	262	1800	CholB16	WelB2	WelB3	WelB5	WelB6	WelB7	WelB22	WelB23	S.Weltevreden	1808	26	365	SAL_XA8578AA
21	en8	1450	STMB0	STMB32	STMB1	STMB5	STMB6	STMB7	STMB14	STMB15	*S*.4,5,12:i:-	1686	23	34	SAL_XA8579AA
22	en11	1600	STMB0	STMB32	STMB1	STMB5	STMB6	STMB7	STMB15	STMB16	*S*.4,5,12:i:-	1686	24	34	SAL_XA8579AA
23	L17	1700	STMB0	STMB32	STMB1	STMB5	STMB6	STMB7	STMB13	STMB14	*S*.Typhimurium	1809	26	19	SAL_CB8211AA
24	L27	1700	STMB0	STMB32	STMB1	STMB5	STMB6	STMB7	STMB13	STMB14	*S*. Typhimurium	1809	26	19	SAL_CB8211AA
25	m133	1800	EntB39	IndB1	KenB1	KenB5	KenB6	KenB7	KenB59	KenB20	*S*.Kentucky	1809	26	696	SAL_XA8595AA
26	L8	2000	AgoB1	WorB1	WorB2	EntB40	TenB2var1	9	16	17	*S*.Krefeld	2173	32	3157	SAL_YA8552AA
	Unknown										**Conventional Typing**				
27	482	1000	STMB0	STMB32	STMB1	STMB21	STMB22	STMB23			*S*.Typhimurium	1075	14	19	SAL_LA4069AA
28	466	1000	STMB0	2	3	BovB5	BovB6	BovB7			*S*.Bovismorbificans	1076	14	1499	SAL_FA5988AA
29	443	2100	AgoB1	WorB1	WorB2	EntB40	TenB2var1	9			*S*.Krefeld	2173	32	746	SAL_YA8552AA
30	475	2100	AlbB3	AlbB4	Kot26	7					*S*.Albany	2173	32	292	SAL_XA8577AA
31	411	850	PanB1	ParAB0var1	PanB2	PanB3	PanB4	PanB5			*S*.Panama	773	9	48	SAL_ZA4097AA
32	483	1600	ParAB0	InfB1	NewpB1	NewpB11	NewpB43	NewpB13			*S*.Newport	1501	NA	NA	NA
33	495	450	EntB0	IndB1	EntB10	4					*S*.Stanley	NA	NA	NA	NA
34	454	1800	CholB16	WelB2	WelB3	WelB5	WelB6	WelB7			*S*.Weltevreden	1808	26	365	SAL_XA8578AA

Note: *#C2 fragments: PCR amplicons from CRISPR 2 (C2) amplification (base pair: bps), $s(n): the abbreviated name of spacer from 5' of CHRIST 2 locus in sequential order of the number, expected total number of spacers C2, Expected ST: multi locus sequence type (MLST) derived from corresponding WGS data analysis, WGS reference from enterobase.com, NA: not analyzed, Number symbol [1-17] in spacer column defile new spacers (not in database).*

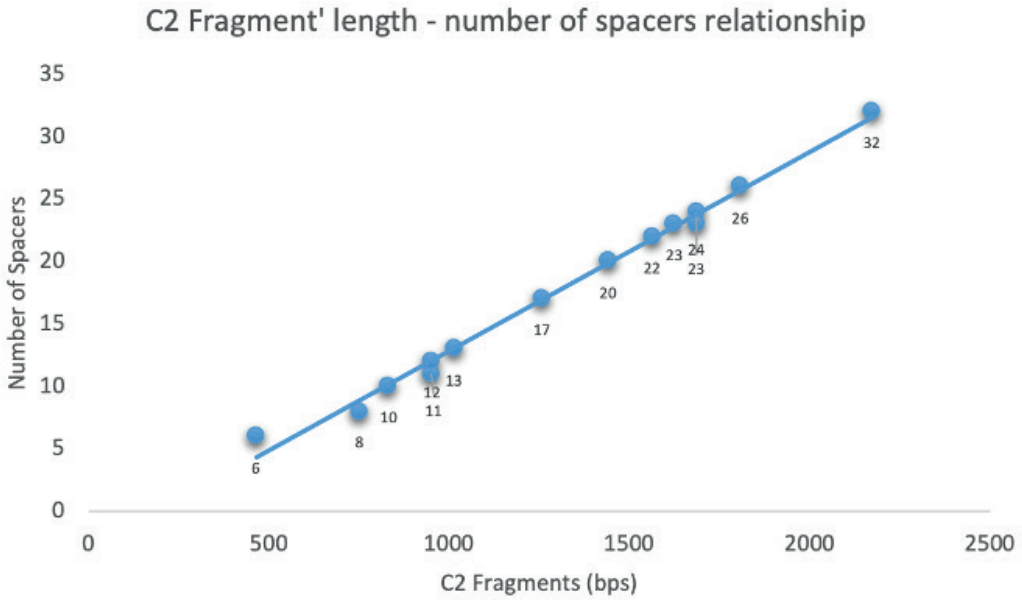

Figure 3 *Graph showed a linear relationship between the length of CRISPR 2 (C2) fragments (bps) and the total number of spacers observed from 14 Salmonella serotypes from various sources in northern Thailand (2015-2017).*

***Salmonella* Virulotypes for Subgrouping**

The study analyzed seven virulent factors in *Salmonella*, including fimbrial genes (*bcf*C, *csg*A, *agf*A), the invasive gene *inv*A, and bacteriophage-related genes (*gip*A, *sop*E1, *sod*C1), employing uniplex or multiplex PCR. Most isolates exhibited *inv*A, *bcf*C, and *csg*A, with exceptions in *S*. Montevideo, *S*. Enteritidis, *S*. Give, and *S.* Derby lacking one or more of these genes (Table 4). Interestingly, serotypes such as *S.* 4,5,12:i-, *S.* Typhimurium, and *S.* Enteritidis demonstrated unique patterns in *agf*A, *gipA*, *sop*E1, and *sod*C1, aiding further subtyping. For instance, the majority of *S.* 4,5,12:i- isolates presented two dominant virulent profiles, mainly distinguished by the presence or absence of *sop*E1. *S.* Typhimurium and *S.* Enteritidis also showed distinct gene patterns, notably in *agf*A and *gip*A.

Table 4 Virulent gene distribution, CRISPR 2 amplicons and Virulotypes of 17 *Salmonella* serotypes and 8 unknowns from various sources in northern Thailand (2015-2017).

Serotypes	Number of strains (N)	Clade	CRISPR 2 (bps)	Virulotypes	Virulent genes						
					*Inv*A	*bcf*C	*csg*A	*agf*A	*sod*C1	*Sop*E1	*gip*A
*S.*4,5,12:i:-	9	1	1600	V_1	■	■	■	■	■		■
	1	1	1550	V_1	■	■	■	■	■		■
	8	1	1600	V_2	■	■	■	■	■	■	■
	2	1	1600	V_3	■	■	■	■	■	■	
	1	1	1600	V_4	■	■	■	■	■		
					100%	100%	100%	100%	100%		
*S.*Typhimurium	2	3	1700 bp	V_1	■	■	■	■	■		■
	1	3	1700 bp	V_5	■	■	■		■		■
					100%	100%	100%		100%		100%
*S.*Enteritidis	3	3	800 bp	V_6	■	■	■	■	■	■	
	2	3	800 bp	V_7	■	■	■		■	■	
	1	3	800 bp	V_8	■	■	■		■	■	■
					100%	100%	100%		100%	100%	
*S.*Weltevraden	2	3	1650	V_5	■	■	■		■		■
	1	3	1500	V_1	■	■	■	■	■		■
	1	3	1600	V_14	■	■	■				■
					100%	100%	100%				100%
*S.*Stenley	1	3	1500	V_9	■	■	■			■	■
	1	3	1500	V_10	■	■	■				■
	1	3	1600	V_11	■	■	■	■			■
					100%	100%	100%				100%
*S.*Derby	1	3	950	V_12	■	■	■			■	
	1	3	900	V_13	■	■	■	■		■	
					100%	100%	100%				
*S.*Agona	1	2	700 bps	V_14	■	■	■				
	1	3	700 bps	V_15	■	■					
					100%	100%					
*S.*Kentucky	1	2	1800	V_16	■	■	■				■
					100%	100%	100%				
*S.*Saintpaul	1	2	1500	V_6	■	■	■	■	■	■	
*S.*Corvallis	1	2	1400	V_14	■	■	■				
*S.*Krefeld	1	2	2000	V_14	■	■	■				
*S.*Braenderup	1	2	ND	V_14	■	■	■				
*S.*Montedeveo	1	2	1550	V_15	■	■					
*S.*Barielle	1	2	850	V_14	■	■	■				
*S.*Kedouko	1	2	900	V_14	■	■	■				
*S.*Give	1	3	450	V_16	■						
*S.*Schwarzengrund	1	2	1300	V_14	■	■	■				
					100%						

Table 4 Virulent gene distribution, CRISPR 2 amplicons and Virulotypes of 19 *Salmonella* serotypes and 8 unknowns from various sources in northern Thailand (2015-2017). *(continued)*

Serotypes	Number of strains (N)	Clade	CRISPR 2 (bps)	Virulotypes	Virulent genes						
					***Inv*A**	***bcf*C**	***csg*A**	***agf*A**	***sod*C1**	***Sop*E1**	***gip*A**
Unknown											
482(*S.*Typhimurium)	1	2	1000	V_2	■	■	■	■	■	■	■
411 (*S.* Panama)	1	2	700	V_8	■	■	■		■	■	■
466 (*S.* Bovismorbificans)	1	2	1000	V_7	■	■	■		■	■	
483 (*S.* Newport)	1	2	1500	V_3	■	■	■	■	■	■	
443 (*S.*Krefeld)	1	3	2000	V_17	■	■	■	■			
475 (*S.* Albany)	1	3	2000	V_17	■	■	■	■			
495 (*S.*Stanley)	1	3	400	V_17	■	■	■	■			
454 (*S.*Weltevraden)	1	3	1900	V_8	■	■	■		■	■	■
					100%	100%	100%				

The analysis revealed that specific virulent gene patterns could significantly differentiate the *S.* 4,5,12:i- isolates into major subtypes, primarily based on *sop*E1 presence. This finding underscores the potential of using virulent gene patterns in *Salmonella* subtyping, especially in understanding the pathogenicity and epidemiology of these serotypes in northern Thailand.

Virulotypes and CRISPR 2 Spacer Profiles in Clinically Important* Salmonella *Serotypes

We analyzed *S.* 4,5,12:i-, *S.* Typhimurium, and *S.* Enteritidis from northern Thailand (2015-2017) for virulent gene profiles and CRISPR 2 spacer patterns. Twenty-one *S.* 4,5,12:i- strains exhibited two dominant virulent gene profiles: one with *sop*E1 (n=10, V_1) and another without *sop*E1 (N=8, V_2). Most of these strains consistently produced a CRISPR 2 fragment of 1600 bps, characterized by a specific spacer architecture (24 spacers; 3' spacer stmB0; 5' spacer stmB33) and MLST type 34 (Table 5). Two *S.* Typhimurium strains shared a virulent gene profile similar to *S.* 4,5,12:i- (V_2), but with a distinct CRISPR 2 fragment length (1700 bps) and spacer architecture (26 spacers; 3' spacer stmB0; 5' spacer stmB36), correlating with MLST type 19. An unknown *S.* Typhimurium strain exhibited a truncated CRISPR 2 fragment (1000 bps) with a different spacer pattern. *S.* Enteritidis strains demonstrated varied virulent gene profiles yet shared a consistent CRISPR 2 fragment length (800 bps) and spacer architecture (10 spacers; 3' spacer entB0; 5' spacer entB9), aligning with MLST type 10.

In summary, combining virulent gene profiling with CRISPR 2 PCR effectively differentiated three clinically significant *Salmonella* serotypes, particularly subtypes of *S.* 4,5,12:i-. This approach shows two subtypes of *S.* 4,5,12:i- circulating in both hospitalized patients and minced pork, facilitating *Salmonella* epidemiological research in northern Thailand.

Table 5. CRISPR 2 – virulent gene-based subtypes of clinically important *Salmonella Typhimurim Enteritidis* and 4,5,12i- isolates from various sources in northern Thailand (2015 -2017).

	Number (N=63)	ID	Source	Virulent genes	Predicted ST type	C2 Fragments (bps)	CRSPR 2 variation	Predicted Number of spacers (C2)	5' Spacer	3' Spacer	WGS data Reference
*S.*4,5,12:i:-	8	25, 345, 392, 412, 458 474, 484	Hospitalized patients	*InvA-bcfC-csgA-agfA-sodC1-sopE1-gipA*	34	1600		24	STMB0	STMB33	SAL_XA8579AA
		en21	Minced pork								
	10	316, 350, 497, 35, 23, 9	Hospitalized patients	*InvA-bcfC-csgA-agfA-sodC1-gipA*		1600		24			
		en11, en26	Minced pork								
		en8	Minced pork			1550	Δ STMB9	23			
		L7	Swine			1600		24			
	2	394, 444	Hospitalized patients	*InvA-bcfC-csgA-agfA-sodC1-sopE1*							
	1	1	Hospitalized patients	*InvA-bcfC-csgA-agfA-sodC1*							
*S.*Typhimurium	4	L27, L28	Chicken, Goat	*InvA-bcfC-csgA-agfA-sodC1-gipA*	19	900		10		STMB31	SAL_CB8211AA
		482 (unknown)	Hospitalized patients	*InvA-bcfC-csgA-agfA-sodC1-sopE1-gipA*							SAL_LA4069AA
		L17	Chicken	*InvA-bcfC-csgA-sodC1-gipA*							SAL_CB8211AA
*S.*Enteritidis	6	49,450,487	Hospitalized patients	*InvA-bcfC-csgA-agfA-sodC1-sopE1*	10	900		10	EntB0	EntB9	SAL_XA8590AA
		402	Hospitalized patients	*InvA-bcfC-agfA-sodC1-sopE1*							
		L24	Minced pork								
		393	Hospitalized patients	*InvA-bcfC-csgA-sodC1-sopE1-gipA*							

Note: *STMB8var1: TGCCAGTGACTACAGAAGCGTCTCTATCGGGG, STMB8var2: TCCCAGTGACTACAGAAGCGTCTCTATCGGGG*

Discussion

Salmonella spp. predominantly affects children and immunocompromised patients, exhibiting varying degrees of virulence, antibiotic resistance, and host specificity associated with different *Salmonella* serovars.[41] *S.* Enteritidis is often linked to bacterial sepsis, while *S.* Typhimurium, including its monophasic forms, frequently leads to prolonged hospitalization due to its high invasiveness and antibiotic resistance.[42] The rapid diagnosis of *Salmonella* serotypes, utilizing advanced molecular tools, is emerging as a highly potential technique for investigating salmonellosis. Various specific endpoint multiplex PCRs have been effectively used to target genes of o-antigens (*wzx*C2, *rfb*J, *prt*, *tyv*, *wzx*E, *wzx*C1, *prt*) and flagella antigens (*fli*C and *flj*B) for typing *Salmonella* serotypes. However, the large number of primers and the complexity of data interpretation often limit their practical application.[43] Alternatively, multiplex PCR combined with High-Resolution Melting (HRM) analysis, targeting sequence polymorphism of three genes, has proven effective in typing several epidemiologically significant *Salmonella* serotypes in Sweden and Thailand.[19,44] Furthermore, machine learning algorithms have been successfully applied to distinguish *Salmonella* serotypes with closely related HRM patterns. CRISPR loci, especially CRISPR 2, have been identified as promising gene targets containing unique DNA signatures capable of specifying S*almonella* subtypes.[45] However, so far, only the CRISPR 2 locus has been developed for characterizing *Salmonella* serotypes through the observation of different DNA amplicons on simple agarose gel electrophoresis.[38] Traditionally, MLVA analysis, typically observed using advanced Capillary Electrophoresis (CE), has been performed to subtype *Salmonella* spp.[46] In this study, however, CRISPR 2 locus analysis, in conjunction with MLVA, was used to generate DNA fingerprint patterns specific to various *Salmonella* serotypes based on agarose gel electrophoresis. To the best of our knowledge, this is the first instance of MLVA analysis being applied in combination with CRISPR 2 PCR to characterize *Salmonella* serotypes using simple agarose gel electrophoresis.

Salmonella enterica serotype 4,5,12:i- is globally prevalent and is often considered to exhibit a high degree of clonal prevalence. Subtyping of *S.* 4,5,12:i- has predominantly been conducted using sophisticated molecular techniques, such as Multilocus Variable-Number Tandem Repeat Analysis (MLVA) with capillary electrophoresis,[46] phage typing, and Pulsed-Field Gel Electrophoresis (PFGE) ,[47] along with CRISPR loci analysis and Whole Genome Sequencing (WGS).[48] Our previous efforts to rapidly subtype *S.* 4,5,12:i- using CRISPR 2 PCR in conjunction with High-Resolution Melting (HRM) analysis achieved limited success.[26] In this study, however, the use of CRISPR 2 and virulent gene amplicons, observed through agarose gel electrophoresis, successfully identified *S.* 4,5,12:i- ST 34 with varying virulotypes in northern Thailand. Genes such as *sop*E1, located on cryptic P2-like prophages, and *gip*A (gipsy-1) have been suggested as markers for subtyping S. 4,5,12:i- ST 34, supporting previous research conducted in China.[49] The heterogeneity of *sop*E1 is likely linked to phage-mediated horizontal gene transfer.[50] Recent research involving a substantial dataset (N=173) focused on the polymorphism of CRISPR 1 and 2, revealing CRISPR type TST4 as the most prevalent subtype of *S.* 4,5,12:i- in pig production in China.[51] Additionally, epidemiological studies have shown that the CRISPR-Cas system, especially the cas genes, used for classifying *S.* Typhi, is associated with varying antimicrobial resistance (AMR) statuses, demographic origins, and endemic isolates in South Asian countries.[51]

In summary, the combination of CRISPR 2 - MLVA analysis and virulotyping provided essential insights into *Salmonella* serotypes. This approach was particularly effective in determining the sequence type (ST) for regional *Salmonella* surveillance. Notably, the majority of *S.* 4,5,12:i- strains were identified as ST 34, with or without the *sop*E1 gene. The prevalence of these subtypes underscores their role in *Salmonella* transmission, contributing to the rate of human infections in northern Thailand between 2015 and 2017. Our future work will focus on using this approach with other serotypes to ascertain its efficiency.

Conclusion

In conclusion, the CRISPR 2 - MLVA analysis, complemented by virulotyping, has been instrumental in identifying and subtyping *S.* 4,5,12:i- in our study region. These methods not only provide preliminary serotype identification but also enable efficient determination of sequence types. This approach has significant implications for understanding *Salmonella* transmission and prevalence in human infections, particularly in northern Thailand between 2015-2017.

Data availability

The raw sequence of CRISPR 2 amplicon of 8 unknowns of *Salmonella* spp. and their CRISPR analyzation using online tool of CRISPRCasMeta [online] https://crisprcas.i2bc.paris-saclay.fr/CrisprCasMeta/Index

Acknowledgements

The authors acknowledge the financial support from a research grant from the Molecular Genetic of Bacterial Pathogen Unit of Excellence (MGBP) numbered FF64-UoE022, University of Phayao. Importantly, the first author wishes to sincerely pay his respects to Prof. Prapon Wilairat, who always supports both knowledge and the passion for mastering knowledge.

Conflicts of Interest

The authors confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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