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High-resolution melting-curve analysis for serotyping of *Salmonella* spp. group B isolated from minced pork in the Northern part of Thailand

Kritchai Pooncharean[1*] Narong Nuanmuang[2] Porntip Prommuang[3] Sukhontip Sriisan[1]

[1]Division of Biochemistry, School of Medical Sciences, University of Phayao, Tambon Maeka, Amphur Muang, Phayao Province, Thailand.
[2]Division of Microbiology, Department of Medical Technology, School of Allied Health Sciences, University of Phayao, Phayao Province, Thailand.
[3]Veterinary Research and Development Center (Upper Northern Region) Lampang Hang Chat, Lampang

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ABSTRACT

Background: Nontyphoidal *Salmonella* spp. is the major bacterial cause of food poisoning. Conventional serotyping is complicated and time consuming.

Objectives: To establish a rapid molecular - based screening for *Salmonella* serotypes

Materials and methods: Several aspects of *Salmonella* isolates were characterized by both rapid multiplex real-time PCR and high-resolution melting-curve (HRM) analysis. Group B *Salmonella* isolates (n=29) were isolated from 165 of minced pork samples randomly collected from local markets in six provinces of the Northern Thailand. Several genetic determinants responsible to specific phenotypes were selected, including *Salmonella* spp. (*Inv*A), *Salmonella* Serotypes (*flj*B, *gyr*B and *ycf*Q), Beta lactam resistance including serious ESBL determinants (*bla*TEM, *bla*CTX-M).

Results: HRM serotyping successfully revealed the epidemiological prevalence of three *Salmonella* serotypes from all group B *Salmonella* isolates, including 38% *S.* Stanley, 24% *S.* Typhimurium, and 17% *S.* Monophasic. Further conventional serotyping showed five unknown HRM patterns as *S.* Agona, *S.* Schwarzengrund, *S.* Saintpaul, *S.* Brandenburg and one unknown serotype. Fifty-five percent of the isolates showed multidrug-resistant phenotype. The high prevalence of *bla*TEM gene totally corresponded to the observed ampicillin-resistant phenotype. However, the presence of *bla*CTX-M group 1 was widely observed but not corresponded to its expected ESBL phenotype. Melt curve analysis of the observed *bla*CTX-M group 1 amplicons compared with the positive ESBL gene (*bla*CTX-M -55) showed the high difference in the melting temperature (T_m) of those amplicons which indicated that the observed *bla*CTX-M group 1 amplicons were less likely to be ESBL gene. Only one ESBL *Salmonella* isolate from Nan province showed the presence of *bla*CTX -M group 9 with ESBL phenotype. The highly virulent ESBL *Salmonella* serovar Typhimurium encoding *bla*CTX-M group 9 in contaminated minced pork from the Nan province suggested the high alert for the rapid screening of ESBL producing *Salmonella* spp. in meat and animals to prevent a potential future outbreak.

Conclusion: By performing the molecular analysis, this study successfully revealed the importantly epidemiological aspects of the *Salmonella* isolates group B collected from the Northern Thailand. This approach should simplify the screening for *Salmonella* serotypes in minced pork.

* **Corresponding author.**
Author's Address: Division of Biochemistry, School of Medical Sciences, University of Phayao, 19 Moo 2, Tambon Maeka, Amphur Muang, Phayao Province, Thailand.
** **E-mail address:** : kof_of@hotmail.com
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Introduction

Nontyphoidal *Salmonella* (NTS) is the major pathogen causing gastroenteritis to mostly young children.[1] Commonly, *Salmonella*-infected victims were initially exposed to the contaminated environment and usually infected by the non-hygienic behaviour of the victims. Nontyphoidal *Salmonella* spp. has been classically classified by the different cellular appearance of somatic antigens (O antigens) combined with flagellar antigens (H antigens). To initially determine *Salmonella* serogroups, only unique O antigen was initially diagnosed to more than 50 distinct groups.[2] For the complete serotyping, the determination of phase 1 and 2 H antigens was executed to derive the serotype or unique antigenic combinations between O and H antigens according to the complete standard *Salmonella* typing system (Kauffmann-White scheme).[2]

In Thailand, the incidence of salmonellosis was documented through the epidemiological survey of both governmental agencies[3] and independent research.[4] The conventional typing of *Salmonella* serotypes was usually performed as the standard technique for identifying *Salmonella* serotypes. The epidemiological results showed that some specific *Salmonella* serogroups predominated in certain area causing regional health problems; nevertheless, different types of samples showed various distribution of some serogroups especially group B which was reported to be the most predominated and virulent serogroup.[4] The distribution of *Salmonella* serovars in Thailand is slightly different throughout various geographical regions[5] but significantly different among continents.[6] In Thailand, the most common serovars of *Salmonella* spp. isolated from humans was *Salmonella* Group B including Typhimurium, Anatum, Derby, and Stanley.[7]

Multi-Drug Resistance (MDR) was observed frequently in the major prevalent nontyphoidal *Salmonella* serovars found in human gastroenteritis and the current occurrence has been increased and related significantly to *Salmonella* spp. group B.[4] In Thailand, the significant increase of the second-line antibiotic ceftriaxone, amikacin and kanamycin resistance was noticed and could possibly be related to the over-use of antibiotics to livestock feed and medical treatment.[8] In addition, the resistance to the effective quinolone drugs such as nalidixic acid was also reported.[9] The failure of antibiotic treatment from nearly all third-generation cephalosporin antibiotics, classified as Extended Spectrum β-Lactamases (ESBLs) has created the major threat to the unresponsive cases toward the first and second line therapies which currently has been the subject of active clinical epidemiological research.[10] ESBL genes include those encoding various beta-lactamases such as the classical *bla*TEM, *bla*SHV and *bla*OXA group and the newer *bla*CTX-M, *bla*CMY and *bla*DHA group usually observed with different levels of virulence and epidemiological capability of transmission.[11] ESBL *Salmonella* spp. encoding *bla*CTX-M are of concern due to their capability for global dissemination through both clonal and horizontal transfer of the gene.[12]

The standard method for *Salmonella* identification is based on the culture method recommended by ISO system.[13] The assay employs different selective media to identify *Salmonella* spp. and then performs serotyping by serological identification based on different combinations of O and H antigenic determinants.[1] The method requires skilled personnel to perform the standardized protocols, which are quite laborious and time-consuming. In order to reduce the turnaround time for subtyping *Salmonella* spp., multiplex PCR of specific gene determinants was introduced.[14] The molecular subtyping for *Salmonella* serovar identification was based on the sequence polymorphism of *rfb* locus and flagellar alleles as gene targets.[15] Other molecular modifications such as High - resolution melting - curve analysis has been coupled to the multiplex PCR for detection of polymorphisms from 16S rDNA[16], *flj*B, *gyr*B and *ycf*Q. [17]

The objective of this study was to reduce operational cost and turnaround time of the traditional culture assay by performing the rapid molecular-based assays of sufficient efficacy for analyzing some important epidemiological data from 29 *Salmonella* spp. isolates group B in 165 minced pork collected from local grocery stores of five provinces in the Northern part of Thailand during June to October 2017. In this study, *Salmonella* serotyping was performed by the multiplex HRM serotyping together with the conventional multiplex PCR for the identification of common beta-lactamase genes corresponding to ampicillin and Extended Spectrum β-Lactamases ESBL phenotype.

Materials and methods

Sample collection and Salmonella isolation and identification

The total of 29 *Salmonella* species group B isolated and identified as followed. Briefly, a total of 165 minced pork samples was randomly collected from retail markets of five provinces in the Northern part of Thailand during June to October 2017. All samples were collected in transport media and carefully kept at 4 °C until further isolation and identification process at the University of Phayao. The samples were then transferred to buffered peptone water (BPW; Oxoid, Hampshire, UK) with overnight incubation at 37 °C, later transferred to both TT broth and RVS broth (Oxoid, Hampshire, UK) with overnight incubation at 37 C° and 42 C° respectively. Both overnight TT and RVS were separately plated on XLD agar (Oxoid, Hampshire, UK) and incubated overnight at 37 C°. Black centre dot colonies referred as suspected *Salmonella* colonies were picked to perform 2 biochemical tests; triple sugar iron (TSI) slant, and motility indole lysine agar (MIL) (Biomedia, Nonthaburi, Thailand). The positive colonies were selected to identify serogroups by using *Salmonella* O Polyvalent A-I Group: A,B,C,D,E,F,G,H,I (S&A REAGENTS LAB LTD, Thailand)

Determination of antibiotic-resistance profile

Susceptibility to antibiotics was performed using the disk diffusion method of the Clinical and Laboratory Standards Institute (CLSI)[18] with Ampicillin (AMP) l0 µg, Amoxicillin-clavulanate (AMC) 20-10 µg, Ceftazidime (CAZ) 30 µg, Cefotaxime (CTX) 30 µg, Cefepime (FEP) 30 µg, Ceftriaxone (CRO) 30 µg, Nalidixic acid (NA) 30 µg, Chloramphenicol (C) 30 µg, Streptomycin (S) 10 µg and Sulphamethox/trimethoprim (SXT) 1.25 µg/23.75 µg, Tetracycline (TE) 10 µg (Oxoid, Hampshire, UK). *Escherichia coli* ATCC 25922 was used as a negative control strain. ESBL test was performed

using the combination disk method according to CLSI criteria[18] with both ceftazidime (30 µg), cefotaxime (30 µg) alone and combined with clavulanic acid (10 µg) (Oxoid, Hampshire, UK). In-house known ESBL-producing *Escherichia coli* and ESBL-negative *Escherichia coli* strains ATCC 25922 were used as controls.

Determination of *Salmonella* serotypes

Determination of *Salmonella* serotypes by multiplex PCR coupled with High-resolution melting-curve analysis was initially performed with the DNA extraction from *Salmonella* isolates as previously described.[19] In brief, 1 mL aliquot of an overnight culture was centrifuged at 8,000 rpm for 2 minutes and the pellet was washed twice with 400 µL of STE buffer (10 mM Tris HCl, pH 8.0, 1 mM EDTA and 100 mM NaCl) and resuspended in 200 µL of TE buffer (10 mM Tris HCl, pH 8.0 and 1 mM EDTA). 100 µL of Tris HCl pH 8.0-saturated phenol solution was added, mixed and centrifuged at 13,000 rpm for 5 minutes at 4°C. The 160 µL aliquot of the upper aqueous layer was mixed with 40 µL of TE buffer and 100 µL of chloroform and then centrifuged for 5 minutes at 13,000 rpm at 4°C. The upper aqueous solution was extracted with chloroform and 150 µL aliquot was kept at -20°C until used. Multiplex PCR coupled with High-resolution melting-curve analysis was performed using a combination of primers to amplify *flj*B (170 bps), *gyr*B (171 bps) and *ycf*Q (241 bps) (Table 1). Multiplex PCR coupled with High - resolution melting - curve analysis was conducted in BIORAD CFX96™ Real-Time System (Bio-Rad, Hercules, CA, USA). The multiplex PCR mixture (10 µL) contained 1 µl of DNA, 0.1 pmol of *gyr*B, 0.075 pmol of *flj*B and 0.075 pmol of *ycf*Q primer pairs and 2 µL of HOT FIREPol EvaGreen: no ROX Mix (Solis Biodye, Tartu, Estonia). Thermocycling conditions were as follows: 95°C for 15 minutes, followed by 45 cycles of 95°C for 10 sec, 60°C for 10 sec and 72°C for 20 sec. Samples were then heated at 95°C for 1 minute, cooled to 40°C for 1 minute and High-resolution melting-curve analysis was performed from 70°C to 95°C, rising at 0.2°C/s, with 25 acquisitions per degree Celsius. HRM profile was generated using the Precision Melt Analysis software V 1.2 with the sensitivity setting at 0.30, temperature shift at threshold 5, pre-melt normalization range from 80.87°C to 81.51°C, and post-melt normalization range from 89.17 °C to 89.92°C. Following normalizing and temperature shifting of the melting curves, difference plots were generated by selecting HRM cluster 9, representing *S*. Bareilly as the baseline

Molecular analysis of major beta-lactamase genes

Amplifications of different *bla* alleles were performed by conventional monoplex or multiplex PCR using the primers (IDT, Singapore) listed in Table 1. The reaction mixture (10 µL) contained 1 µL of DNA, primer sets at concentration listed in Table 1 and 2 µL of HOT FIREPol Blend Master Mix Plus 10 mM $MgCl_2$ (Solis Biodye). In multiplex PCR 1 and 2, thermocycling was as follows: 95°C for 12 minutes; 40 cycles of 95°C for 40 sec, 60°C for 40 sec and 72°C for 1 minute; and the final step at 72°C for 7 min. Amplicons were visualized following 1.5% agarose gel electrophoresis by staining RedSafe dye (INiRON, Washington, United States). Data analysis for descriptive statistics was performed by using SPSS for Windows, version 10 (SPSS Inc, Chicago, USA) at the University of Phayao.

Table 1. Primers used in this study[17, 36, 37]

Primer	Genes	Sequence (5'⟶ 3')	Size of PCR- product (bps)	Primer Concentration (pmol/ul)	References
HRM Multiplex *flj*B, *gyr*B and *ycf*Q genes (HRM-rt PCR)					
fljB_f	*flj*B	GTGAAAGATACAGCAGTAACAACG	170	0.075	(17)
fljB_r		ACAAAGTACTTGTTATTATCTGCG		0.075	(17)
gyrB_f	*gyr*B	AAACGCCGATCCACCCGA	171	0.1	(17)
gyrB_r		TCATCGCCGCACGGAAG		0.1	(17)
ycfQ_f	*ycf*Q	GCCTACTCTCTATGCGGAATTCAC	241	0.075	(17)
ycfQ_r		GATATCGCGCGAGGAGGCG		0.075	(17)
Multiplex 1 *bla*TEM variants including *bla*TEM-1 and *bla*TEM-2, *bla*SHV variants including *bla*SHV-1, *bla*OXA-1-like including *bla*OXA-1, *bla*OXA-4 and *bla*OXA-30					
blaTEM_f	*bla*TEM	CATTTCCGTGTCGCCCTTATTC	800	0.4	(36)
blaTEM_r		CGTTCATCCATAGTTGCCTGAC		0.4	(36)
blaSHV_f	*bla*SHV	AGCCGCTTGAGCAAATTAAAC	713	0.4	(36)
blaSHV_r		ATCCCGCAGATAAATCACCAC		0.4	(36)
blaOXA_f	*bla*OXA	GGCACCAGATTCAACTTTCAAG	564	0.4	(36)
blaOXA_r		GACCCCAAGTTTCCTGTAAGTG		0.4	(36)

Table 1. Primers used in this study[17, 36, 37] (continued)

Primer	Genes	Sequence (5'⟶3')	Size of PCR- product (bps)	Primer Concentration (pmol/ul)	References
Multiplex 2 *bla*CTX-M group 1 and group 9 : variants of *bla*CTX-M group 1 including *bla*CTX-M-1, *bla*CTX-M-3 and *bla*CTX-M-15 : variants of *bla*CTX-M group 9 including *bla*CTX-M-9 and *bla*CTX-M- 14					
CTX 1_f	*bla*CTX-M group 1	TTAGGAARTGTGCCGCTGYA[b]	688	0.4	(36)
CTX 1_r		CGATATCGTTGGTGGTRCCAT[b]			(36)
CTX 9_f	*bla*CTX-M group 9	TCAAGCCTGCCGATCTGGT	561	0.2	(36)
CTX 9_r		TGATTCTCGCCGCTGAAG			(36)
Uniplex 1 *Inv*A specific to *Salmonella* spp.					
InvA_f	*Inv*A	GTGAAATTATCGCCACGTTCGGGCAA	284	0.125	(37)
InvA_r		GCCCCGGTAAACAGATGAGTATTGA			

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

Results

HRM serotyping of the 29 *Salmonella* isolates group B precisely revealed three dominated *Salmonella* serotypes and five unknown *Salmonella* serotypes

All 29 isolates of *Salmonella* spp. group B were the isolates from Chiang Mai (n=4), Chiang Rai (n=5), Lampang (n=4), Nan (n=2), Phrae (n=2) and Phayao Province (n=12). To perform rapid molecular typing, the HRM serotyping was performed including the real-time multiplex PCR and then followed by the High-Resolution Melting-curve analysis (HRM). HRM patterns of all *Salmonella* isolates showed principal T_m at 87 °C but some additionally produced T_m at 83 °C. To generate their unique melt curves for *Salmonella* typing, the process of melt curve analysis was performed primarily by the auto-clustering function of the machine which effectively differentiated eight unique clusters with high confidence (>99.0%) except CM 28.1 isolate as shown in Table 2.

Table 2. HRM serotyping of *Salmonella* spp. (n=29) isolated from minced pork collected during June to October 2017 from the Northern part of Thailand

Isolate no.	Isolate name*	HRM Serotyping					
		T_m Peak	Clustering	Percent confidence	Cluster	Predicted Serotyped	Serotype
1	CR6.1	2(87.8,83.6)	Auto	> 99.0	1	Stanley	Stanley
2	CR7.1	2(87.6,83.6)	Auto	> 99.0	1	Stanley	Stanley
3	CR10.1	2(87.6,83.4)	Auto	> 99.0	1	Stanley	Stanley
4	CR11.1	2(87.6,83.4)	Auto	> 99.0	1	Stanley	Stanley
5	CR17.1	2(87.6,83.6)	Auto	> 99.0	1	Stanley	Stanley
6	PY5.1	1(87.4)	Auto	> 99.0	2	Typhimurium	Typhimurium
7	PY6.1	2(87.6,83.6)	Auto	> 99.0	1	Stanley	Stanley
8	PY11.1	1(87.2)	Auto	77.1	2	Typhimurium	Typhimurium
9	PY13.1	2(87.6,83.4)	Auto	> 99.0	1	Stanley	Stanley
10	PY21.1	2(87.6,83.4)	Auto	> 99.0	1	Stanley	Stanley
11	NA6.1	1(87.2)	Auto	98..2	3	Monophasic	Monophasic
12	NA14.1	1(87.6)	Auto	> 99.0	2	Typhimurium	Typhimurium
13	CM16.1	1(87.4)	Auto	> 99.0	2	Typhimurium	Typhimurium
14	CM21.1	1(87.6)	Auto	72.1	6	**Unknown**	**Agona**
15	CM28.1	2(87.6,83.4)	Manual	N/A	1	Stanley	Stanley
16	CM35.1	1(87.4)	Auto	> 99.0	2	Typhimurium	Typhimurium
17	LP4.1	2(87.6,83.4)	Auto	N/A	5	**Unknown**	**Schwarzengrund**
18	LP12.1	2(87.0,83.4)	Auto	98.5	7	**Unknown**	**Saintpaul**
19	LP13.1	1(87.8)	Auto	> 99.0	4	**Unknown**	**Brandenburg**
20	LP17.1	1(87.4)	Auto	98.7	3	Monophasic	Monophasic
21	FM11.1	1(87.2)	Auto	94.8	3	Monophasic	Monophasic

Table 2. HRM serotyping of *Salmonella* spp. (n=29) isolated from minced pork collected during June to October 2017 from the Northern part of Thailand (continued)

Isolate no.	Isolate name*	HRM Serotyping					
		T_m Peak	Clustering	Percent confidence	Cluster	Predicted Serotyped	Serotype
22	FM19.1	1(87.2)	Auto	> 99.0	8	**Unknown**	**Unknown**
23	FM25.1	1(87.4)	Auto	> 99.0	2	Typhimurium	Typhimurium
24	FM30.1	2(87.8,83.6)	Auto	> 99.0	1	Stanley	Stanley
25	FM31.1	1(87.4)	Auto	> 99.0	4	**Unknown**	**Brandenburg**
26	FM32.2	1(87.4)	Auto	98.3	3	Monophasic	Monophasic
27	FM35.1	2(87.8,83.6)	Auto	> 99.0	1	Stanley	Stanley
28	PR5.1	1(87.4)	Auto	> 99.0	2	Typhimurium	Typhimurium
29	PR20.1	1(87.2)	Auto	98.5	3	Monophasic	Monophasic
Positive control (Standard serotyping)		2(87.8,83.6)	Auto	> 99.0	1	Stanley	Stanley
		1(87.2)	Auto	94.8	2	Typhimurium	Typhimurium
		1(87.4)	Auto	> 99.0	3	Monophasic	Monophasic
Reference		1(87.4)	Auto	> 99.0	9	Barille	Barille

** CR: Chiang Rai, PY, FM: Phayao, NA: Nan, CM: Chiang Mai, LP: Lampang, PR: Phrae Province*

Eight unique HRM patterns easily differentiated by visual differentiation were created based on their serotypes. Despite the high degree of similarity between *S.* Typhimurium and *S.* Monophasic HRM curves, they could be differentiated in this study as shown in Figure 1, A. In this study, three known serotypes of *Salmonella* group B were performed along the samples as the positive controls. Each positive control generated the HRM patterns which exactly matched the corresponding serotypes of each sample as shown in Figure 1, B. The epidemiological data concerning the serotypes from HRM serotyping showed the prevalence of detected serotypes as followed; *S.* Stanley (n=11, 38%), *S.* Typhimurium (n=7, 24%), *S.* Monophasic (n=5, 17%), and six unknown serotypes (21%) as shown in Figure 2. Further conventional serotyping revealed six unknown serotypes comprising of *S. Agona, S.* Schwarzengrund, *S.* Saintpaul, *S.* Brandenburg (2) and one nontypable isolate.

MDR phenotype was highly observed in *Salmonella* spp. group B especially *S.* Typhimurium and *S.* Monophasic

All *Salmonella* isolates group B (n=29) showed 55% (n=16) of Multi-Drug Resistance (MDR) phenotype. The prevalence of antibiotic-resistant phenotype was as described; ampicillin (n=23, 80%), tetracycline (n=18, 62%), chloramphenicol (n=9, 31%), nalidixic acid (n=5, 17%), sulphamethox/trimethoprim (n=4, 14%) and cefotaxime (n=1, 3%) as shown in Table 3. The ampicillin resistant phenotype highly corresponded to the isolates processing the common *bla*TEM determinant not *bla*SHV or *bla*OXA. Additionally, the highly virulent gene, *bla*CTX-M group 1 and 9, was also detected and expected to be responsible primarily to ESBL phenotype. However, the observed *bla*CTX-M group 1 did not correspond to their ESBL phenotype. Melting curve analysis between observed *bla*CTX-M group 1 amplicons and *bla*CTX-M - 55 ESBL gene as positive control was performed and the result showed the observed *bla*CTX-M group 1 amplicons in this study processing lower T_m (89.4 °C) than the control ESBL gene (*bla*CTX-M - 55) (T_m = 91.0 °C) as shown in Figure 3. The possible explanation might be their difference in amplicon's sequence or length, thus the observed *bla*CTX-M group 1 amplicon probably did not cause the ESBL phenotype like the virulent *bla*CTX-M - 55 ESBL gene. However, only *S.* Monophasic from Nan province containing *bla*CTX-M group 9 showed the virulent ESBL phenotype. The result implied the emergence of ESBL *Salmonella bla*CTX-M group 9 circulated in minced pork and possibly along the line of the minced pork production.

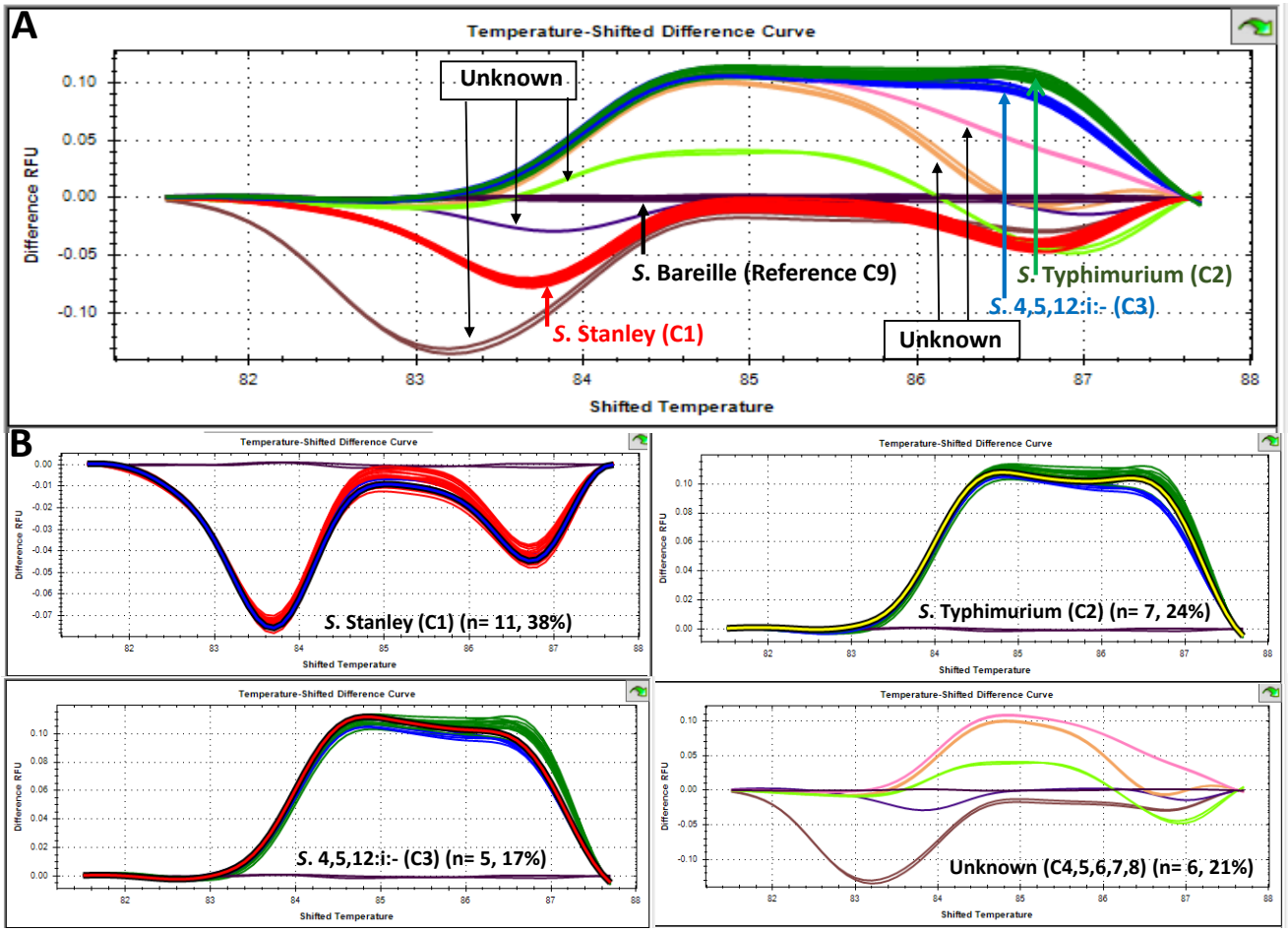

Figure 1 *HRM patterns of all 29 Salmonella isolates from minced pork collected during June to October 2017 from the Northern part of Thailand. (A) All three defined (C1-C3) and five unknown Salmonella serotypes were detected based on their unique HRM patterns. (B) The HRM patterns of each three serotype labeled with both the control (colors and bold line) and samples (colored and fine line) were presented as S.* Stanley *(red, upper left), S.* Typhimurium *(green, upper right) and S.* Monophasic *(blue, lower left). Five unique unknown HRM patterns were presented in lower right.*

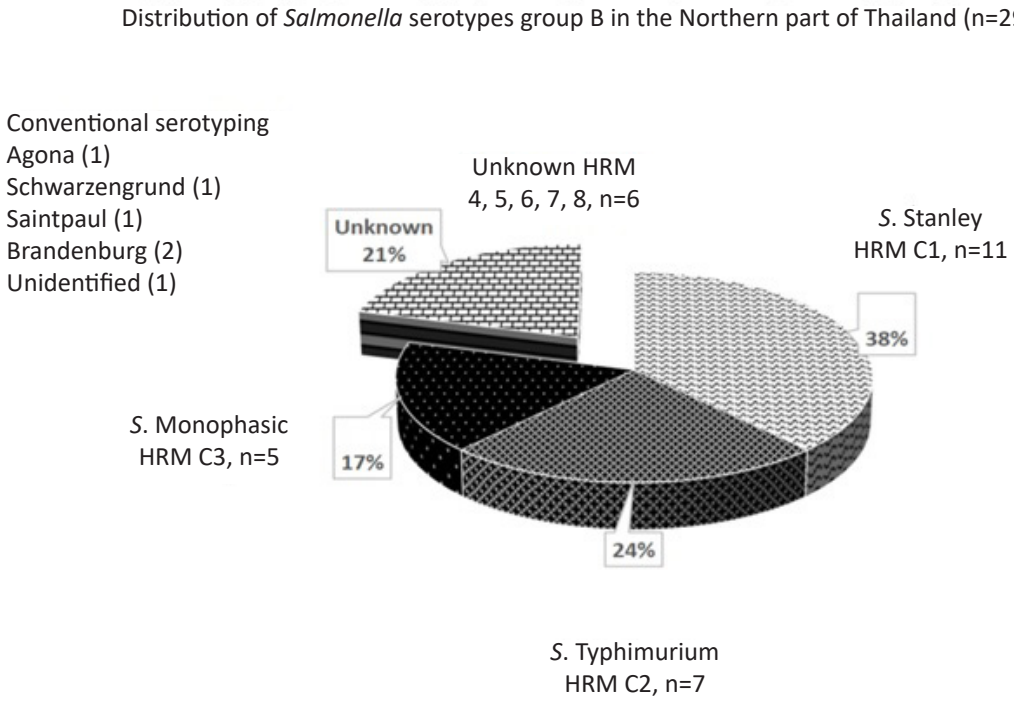

Figure 2 *HRM serotyping representing the epidemiological prevalence of three major serotypes of all 29 Salmonella isolates from minced pork collected during June to October 2017 from the Northern part of Thailand*

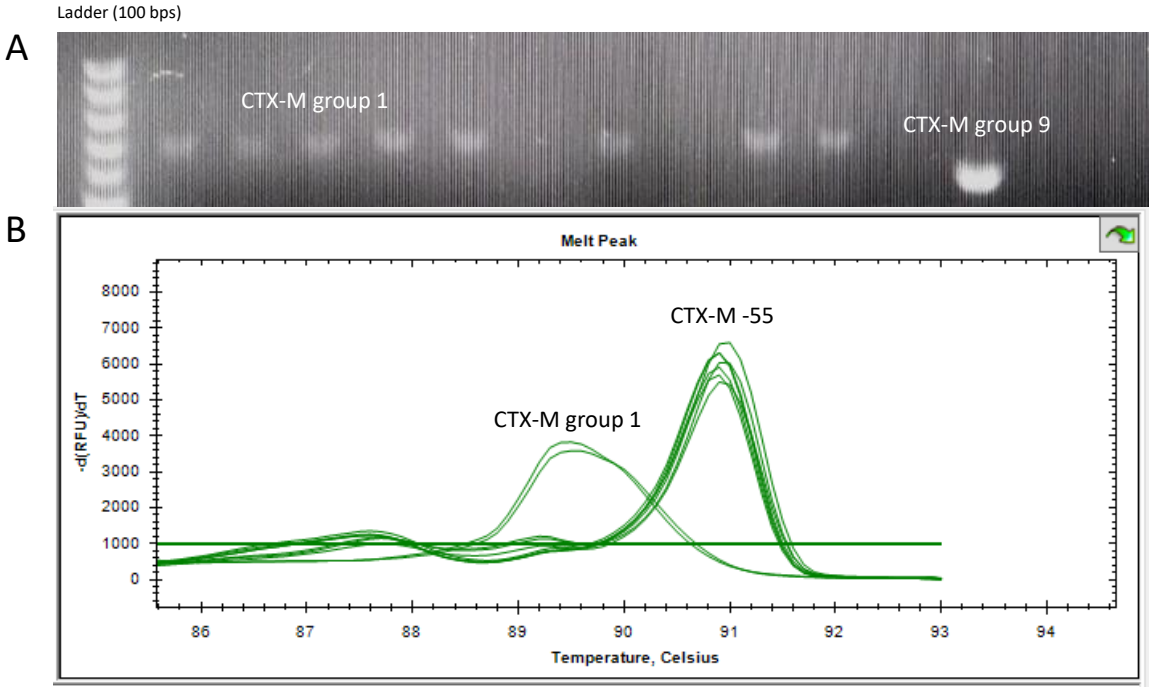

Figure 3 *(A) Multiplex PCR to determine blaCTX-M (ESBL gene) revealed both blaCTX-M group 1 and blaCTX-M group 9 (B) Melt curve analysis of the blaCTX-M group 1 fragments showed the T_m difference between the fragments and the positive control (blaCTX-M-55 or ESBL gene).*

Table 3 Epidemiological details of 29 *Salmonella* isolates from minced pork collected during June to October 2017 from the Northern part of Thailand indicating the important epidemiological data

Province	Serotypes (number of isolates)	Antibiotic-resistant phenotype profile (number of isolates)	Beta-lactam associated genotype	Predicted Beta-lactam phenotype
Chiang Rai	**Stanley[H] (5)**	AMP/TE/NA (3)*	*bla*TEM *bla*CTX-M group 1	AMP
		AMP (2)	*bla*TEM	AMP
Chiang Mai	**Typhimurium[H] (2)**	AMP/TE/SXT/C (2)*	*bla*TEM	AMP
	Stanley[H]	AMP/S/TE*	*bla*TEM *bla*CTX-M group 1	AMP
	Agony[c]	AMP	*bla*TEM	AMP
Nan	**Typhimurium[H]**	AMP/FEP/CTX/CRO/CAZ/TE/C*	*bla*TEM *bla*CTX-M group 9	AMP CTX CRO
	Monophasic[H]	AMP/S/TE*	*bla*TEM	AMP
Phrae	**Typhimurium[H]**	AMP/AMC/TE/SXT/C*	*bla*TEM	AMP
	Monophasic[H]	AMP/AMC/S/TE*	*bla*TEM	AMP
Lampang	**Monophasic[H]**	AMP/S/TE*	*bla*TEM	AMP
	Schwarzengrund[c]	AMP/NA/C*	*bla*TEM	AMP
	Saintpaul[c]	AMP/NA/C*	*bla*TEM	AMP
	Brandenburg[c]	NO	-	NO

Table 3 Epidemiological details of 29 *Salmonella* isolates from minced pork collected during June to October 2017 from the Northern part of Thailand indicating the important epidemiological data (continued)

Province	Serotypes (number of isolates)	Antibiotic-resistant phenotype profile (number of isolates)	Beta-lactam associated genotype	Predicted Beta-lactam phenotype
Phayao	**Typhimurium[H] (4)**	AMP/TE/SXT/C*	*bla*TEM	AMP
		AMP/S/TE/C*	*bla*TEM	AMP
		AMP/TE/C*	*bla*TEM	AMP
		AMP/TE	*bla*TEM	AMP
	Monophasic[H]	AMP/TE	*bla*TEM *bla*CTX-M group 1	AMP
	Stanley[H] (5)	AMP (3)	*bla*TEM *bla*CTX-M group 1	AMP
		AMP/TE (2)	*bla*TEM *bla*CTX-M group 1	AMP
	Brandenburg[c]	No	-	NO
	Unknown	TE	*bla*CTX1	NO

Note: *[H] (HRM serotyping), [c] (Conventional typing), *(MDR), Ampicillin (AMP), Amoxicillin-clavulanate (AMC), Ceftazidime (CAZ), Cefotaxime (CTX), Cefepime (FEP), Ceftriaxone (CRO), Nalidixic acid (NA), Chloramphenicol (C), Streptomycin (S), Sulphamethox/trimethoprim (SXT) and Tetracycline (TE)*

Discussion

The epidemiological research of *Salmonella* spp. is of the ultimate importance and vital procedure to provide sufficient information about the current situation of *Salmonella* prevalence and antibiotic resistance relating to each region and time frame.[20] In Thailand, *Salmonella* isolates from various sources such as clinical samples, contaminated meat, infected animals with geographical difference were collected to assess important epidemiological data such as serotypes, antibiotic-resistant genes; nevertheless, the traditional *Salmonella* serotyping coupled with antibiotic disc diffusion technique needed the long-operational time and complex conventional serotyping.[21] The other rapid modified procedure such as MALDI TOF[22], High - resolution melting - curve analysis[17] and the most resent WGS[23] were efficiently introduced to many epidemiological types of research. High-resolution melting - curve analysis capable of detecting Single Nucleotide Polymorphism (SNP) in amplified PCR products, was introduced to rapidly typing 37 different *Salmonella* serotypes.[17] In Thailand, HRM serotyping was effectively applied for analyzing 14 serotypes from 38 clinical *Salmonella* isolates revealing the most prevalent *Salmonella* spp. as *S.* Stanlay, *S.* Monophasic, and *S. Weltevreden,* respectively.[24] Significantly, *Salmonella* spp. group B or having the common O4 antigen such as *S.* Stanley, *S.* Typhimurium was found to be the predominant serotypes causing invasive salmonellosis which significantly linked to virulent phenotype, multidrug resistance (MDR).[25–27] The epidemiology of *Salmonella* isolated from mince pork in the Northern part of Thailand based on the rapid molecular platform effectively showed the most prevalent serotypes of *Salmonella* group B as *S.* Stanley concentrated mainly in Chiang Rai and Phayao Province and the second and third as *S.* Typhimurium and *S.* Monophasic concentrated mainly in Chiang Mai, Nan, Phrae and Phayao Province corresponding to the *group B Salmonella* isolates in pigs and pork[8,28] as well as in clinical samples.[24] *Salmonella* Typhimurium including variant Monophasic was intensely researched in the epidemiological survey in Thailand[29] and the other countries.[30] *S.* Monophasic was reported to be the cause of the major outbreak in the UK, Spain and Germany and could be separated as an rapidly emerging clade distributed globally possibly due to their capability of fitness to various environments.[31] Additionally, *S.* Typhimurium was reported to be the common serotype showing a high level of virulence and multidrug-resistant pattern.[32] ESBL *Salmonella* producing organisms have been reported to increase in Southeast Asia especially *Salmonella* Typhimurium.[33] The presence of *bla*CTX-M gene was reported to be the common cause of the ESBL phenotype especially *bla*CTX-M group 1 and 9.[34] The acquisition of the ESBL genes was reported through the horizontal transfer from closely related *Escherichia coli.* The ESBL producing *S.* Typhimurium harbouring *bla*CTX-M group 9 was highly observed in food-producing animals in China[35], which might explain its emergence in the Northen part of Thailand. The emergence of the virulent ESBL producing *S.* Typhimurium in Nan province was revealed in this study with the rapid molecular screening or HRM serotyping platform.

In conclusion, the study demonstrates the ability of multiplex PCR coupled with High-resolution melting - curve analysis to rapidly and conveniently serotype *Salmonella* isolates from minced pork . This knowledge and information of the associated antibiogram together with beta-lactamase genes's profile provide a powerful tool for predicting antibiotic resistance of different *Salmonella* serotypes and importantly allow facile monitoring of the emergence of ESBL *Salmonella* spp.

Conflict of Interest

The authors confirm that there are no 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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