

## Feasibility of high resolution melting curve analysis for rapid serotyping of *Salmonella* from hospitalised patients

Kritchai Poonchareon<sup>1</sup> Chaiwat Pulsrikarn<sup>2</sup> Sukon Khamvichai<sup>3</sup> Pakpoom Tadee<sup>4\*</sup>

<sup>1</sup>Division of Biochemistry, School of Medical Sciences, University of Phayao, Phayao Province, Thailand

<sup>2</sup>Department of Medical Sciences, WHO National Salmonella and Shigella Center, National Institute of Health, Ministry of Public Health, Nonthaburi Province, Thailand

<sup>3</sup>Phayao Ram Hospital, Phayao Province, Thailand

<sup>4</sup>Integrative Research Center for Veterinary Preventive Medicine, Department of Food Animal Clinics, Faculty of Veterinary Medicine, Chiang Mai University, Chiang Mai Province, Thailand

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### ABSTRACT

**Background:** Serum agglutination test is the gold standard phenotyping method widely used for *Salmonella enterica* characterisation. This conventional method is limited by its complicated and time-consuming procedures. High resolution melting curve (HRM) analysis is introduced as a rapid and labour-saving method.

**Objectives:** To compare the results of conventional serum agglutination and quantitative PCR-HRM analysis to assess the feasibility of this alternative approach for *Salmonella* serotyping.

**Materials and methods:** *Salmonella* strains from 38 human-originating samples were serotyped using the conventional serum agglutination method and HRM analysis.

**Results:** The conventional serum agglutination assay detected 14 serotypes, while the HRM analysis identified 10 HRM profiles. There was a correlation between most of the serotyping results obtained by the two methods. Nine of the HRM profiles were unique to a single serotype, of each. One exception was HRM\_3. Many of the indistinct curves that were grouped in this HRM pattern belonged to five *Salmonella* serotypes, including Weltevreden, Corvallis, Derby, Kedougou and Kentucky.

**Conclusion:** It is difficult to determine all *Salmonella* serotypes by HRM analysis. However, this method can be used as an alternative to the conventional serum agglutination assay for rapid and labour-saving serotyping.

### Introduction

The foodborne pathogen *Salmonella enterica* causes human gastroenteritis and is considered a world-wide public health problem.<sup>1-3</sup> Contaminated, raw or poorly prepared foods, particularly those originating fresh from farms, are involved in most cases.<sup>4</sup> Characterization of the organism is an essential part of epidemiological surveillance and

outbreak investigation. Serotyping is the most common and simple characterization method in use.<sup>5,6</sup> More than 2,500 serotypes of *S. enterica* have been identified,<sup>7</sup> and the disease appears to be spreading. For example, *Salmonella* Typhimurium and Rissen are the main serotypes in pigs,<sup>1</sup> while *S. Enteritidis* and *S. Dublin* are the main serotypes in poultry and dairy products, respectively.<sup>4,8,9</sup> Seroprevalence data could be used as baseline information to manage and control the disease.

Conventionally, the gold standard serotyping method is based on the Kauffmann-White classification scheme, which involves combining the cell wall O and H antigenic determinations obtained from the agglutination test with matching antiserum.<sup>5,10</sup> However, this method is time

\* Corresponding author.

**Author's Address:** Integrative Research Center for Veterinary Preventive Medicine, Department of Food Animal Clinics, Faculty of Veterinary Medicine, Chiang Mai University, Chiang Mai Province, Thailand

\*\* E-mail address: [td.pakpoom@gmail.com](mailto:td.pakpoom@gmail.com)

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consuming and requires technical skills. The ISO 2002<sup>11</sup> recommends that samples be cultured for at least a week before agglutination is performed. Large numbers of specific sera are also required. To overcome these limitations, high resolution melting curve (HRM) analysis is introduced.<sup>2,12</sup> HRM is a post-quantitative PCR method used to identify variations in gene polymorphism. Serotype recognition based on the differences in PCR melting curves is generated by real-time PCR,<sup>13</sup> providing a fast workflow and high throughputs of data.<sup>2,12</sup> For *Salmonella* serotyping, polymorphisms of the *fljB*, *gyrB* and *ycfQ* genes have been targeted.<sup>13</sup>

The objective of this study was to compare *Salmonella* serotyping obtained from quantitative PCR-HRM analysis and conventional serum agglutination. The results will demonstrate the feasibility of an alternative serotype screening method.

## Materials and methods

### Salmonella strains and serum agglutination tests

A total of 38 *Salmonella* strains were isolated from stool samples of hospitalised patients from the Phayao Ram Hospital, Phayao Province, Thailand from March 2015 to June 2016. The study was conducted under ethical approval (No. 57 02 04 0020) granted by the Ethics Committee of the University of Phayao. Stool samples were initially identified following the ISO protocol.<sup>13</sup> Then, the presumptive *Salmonella* colonies were confirmed with biochemical and serum agglutination tests at the WHO National *Salmonella* and *Shigella* Center, the National Institute of Health, Ministry of Public Health, Nonthaburi Province, Thailand.

### HRM analysis serotyping

For each sample, DNA extraction was completed following the method previously described by Cheng and Jiang.<sup>14</sup> In brief, a 1 mL aliquot of an overnight culture was centrifuged at 8,000 g for 2 min. 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 re-suspended in 200 µL of TE buffer (10 mM Tris HCl, pH 8.0, and 1 mM EDTA). Then, 100 µL of Tris HCl (pH 8.0) saturated phenol solution was added to the suspension, which was subsequently mixed and cen-trifuged at 13,000 g for 5 minutes at 4°C. A 160 µL aliquot of the upper aqueous layer was mixed with 40 µL of TE buffer and 100 µL of chloroform and centrifuged for 5 minutes at 13,000 g at 4°C. The upper aqueous solution was extracted with chloroform, and a 150 µL aliquot was kept at -20°C until used.

Multiplex HRM analysis was performed using a combination of primers to amplify the following genes: *fljB* (5' GTGAAAGATACAGCAGTAACAACG 3' and reverse 5' GCGTCTATTATTGTTTCATGAAAC 3'), *gyrB* (5' AAACGCCGATC-CACCCGA 3' and reverse 5' GAAGGCACGCCGCTACT 3') and *ycfQ* (5' GCCTACTCTCTATGCGGAATTAC 3' and reverse 5' GCGGAGGAGCGCGCTATAG 3'). Sizes of the targeted genes were 170, 171 and 241 bps, respectively. Real-time PCR was conducted using a BIO-RAD CFX96TM real-time system (Bio-Rad, Hercules, CA, USA). The HRM master mixture

(10 µL) contained 1 µL of DNA, 0.1 pmol of *gyrB* primer pairs, 0.075 pmol of *fljB* primer pairs, 0.075 pmol of *ycfQ* primer pairs and 2 µL of HOT FIRE Pol EvaGreen no ROX mix (Solis Biodye, Tartu, Estonia). The thermocycling conditions were programmed according to the protocol of Zeinzing et al.<sup>13</sup> The HRM profiles were generated based on the temperature shift of the melting curves, using *S. Bareilly* as a baseline reference.

### Data analysis

Simpson's diversity index was used to estimate the discriminatory power of the two serotyping methods. In the index range, a minimum value of zero represents no power to discriminate any type in the sample population.<sup>15</sup> Maximum value of one denotes the ability to distinguish all samples in a population. The predictive values of the HRM analysis and the conventional serum agglutination test were evaluated using the Wallace coefficient. This coefficient indicates the probability that a pair of strains assigned to the same type by one typing method is also typed as identical by another method. In the range of 0-1, the maximum value of 1 indicates 100% predictability of one method to another.<sup>16</sup>

## Results

Of the 38 *Salmonella* strains tested, fourteen serotypes were detected by serum agglutination, with the most frequency of *Salmonella* 4, 5, 12 : i : - (12 strains; 32%), followed by *S. Stanley* (9 strains; 23%) and *S. Weltevreden* (5 strains; 13%), respectively (Figure 1). While, the HRM analysis created nine unique HRM patterns corresponding to *S. 4,5,12:i:-*, *S. Stanley*, *S. Newport*, *S. Montevideo*, *S. Enteritidis*, *S. Give*, *S. Yoruba*, *S. Bareilly*, *S. 9,12:-:1,5*. However, HRM\_3 demonstrated several un-specified curves, which were regarded in 5 strains of *S. Weltevreden* and a strain of each belonging *S. Derby*, *S. Corvallis*, *S. Kentucky* and *S. Kedougou* (Figure 2).

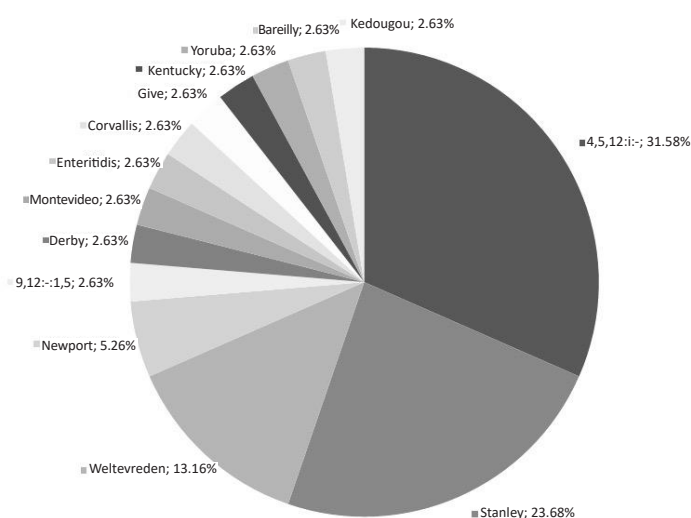


**Figure 1.** Sero-distribution of *Salmonella* strains isolated from hospitalised patients in Phayao Ram Hospital during March 2015 to June 2016 (serum agglutination test)

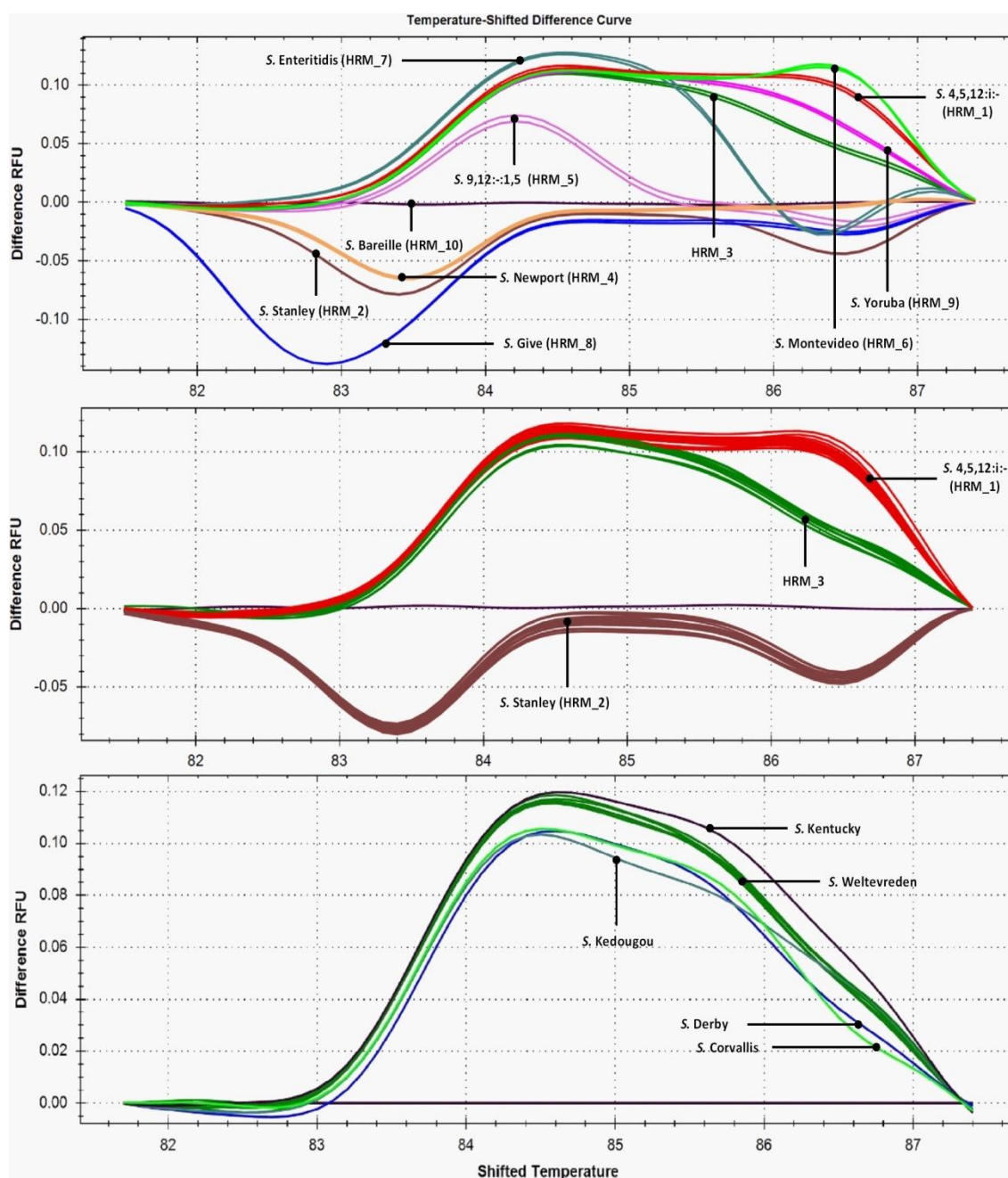


**Figure 2.** HRM profiles of *Salmonella* spp. serotypes following multiplex PCR amplification of *fljB*, *gyrB* and *ycfQ*. Top panel: 10 HRM profiles of all *Salmonella* tested (assigned HRM\_1 to HRM\_10). Middle panel: 3 HRM profiles (HRM\_1 to HRM\_3) in extended RFU scale range. Bottom panel: HRM curves of HRM\_3 in ultra-extended RFU scale range.

Simpson's diversity index indicates a power of distinguishability of the strains in population. The values of two serotyping methods were demonstrated as 0.839 (95% CI; 0.820-0.858) and 0.802 (95% CI; 0.781-0.823) for conventional

serum agglutination and HRM analysis, respectively. For the point of Wallace coefficient measurement, value of HRM analysis to the conventional serotyping was 0.813 (95% CI; 0.730-0.896) (Table 1).

**Table 1.** Simpson's diversity index and Wallace coefficients with the 95% confidence intervals of two serotyping methods.

| Serotyping method | Simpson's index<br>(95% CI) | Wallace coefficients (95% CI) |                         |
|-------------------|-----------------------------|-------------------------------|-------------------------|
|                   |                             | Conventional                  | HRM-analysis            |
| Conventional      | 0.839<br>(0.820-0.858)      | -                             | 1.000*<br>(1.000-1.000) |
| HRM analysis      | 0.802<br>(0.781-0.823)      | 0.813**<br>(0.730-0.896)      | -                       |

\* Wallace coefficients of conventional serotyping method to HRM analysis

\*\* Wallace coefficients of HRM analysis to conventional serotyping method

## Discussion

*Salmonella* is an important foodborne zoonotic pathogen, and it recognized as one of public health concerns, worldwide.<sup>4,7,12</sup> In Thailand, from the surveillance of Bureau of Epidemiology in 2017, an approximate of food poisoning cases in 77 provinces was 108,153 cases or 165.30 per 100,000 of Thai population. In addition, salmonellosis is considered as the top three organisms involving to the problem for a decade, indicating that the disease has been a significant and rising problem in the country.<sup>17</sup>

Thirty-eight *Salmonella* strains recovered from stool of hospitalised patients in Phayao Ram hospital during March 2015 to June 2016, fourteen serotypes were identified by gold standard serum agglutination method. *Salmonella* 4, 5, 12 : i : - is the most frequently detected, followed by S. Stanley and S. Weltevreden, respectively. Considered in the serotype and their potential origins belonged, an antigenic formula of *Salmonella* 4, 5, 12 : i : - is likely allied with S. Typhimurium and S. 4,5,12:i:1,2. Absent of second phase in flagella antigens could be explained in its monophasic variant.<sup>18</sup> Moreover, clonal genetic relatedness among S. 4, 5, 12 : i : - and S. Typhimurium have been identified in pig-associated samples.<sup>19</sup> Besides, sharing of DNA fingerprinting pattern among human-originated and pig-originated *Salmonella* Stanley is also noticed.<sup>20</sup> For S. Weltevreden, it was reported as the most frequently found from chicken meats and human patients in Thailand.<sup>4,21</sup> As a result, pork and poultry consumption are potential reflected as the main source of human salmonellosis in the province.

HRM analysis serotyping has been applied in field practice, recently. A turnaround time of 8-12 hours is approximated. That fewer than a performing in conventional standard method, which is carried out for 3-5 days.<sup>22</sup> One hundred specificity result was proved by Ren et al. (2017), which was performed in S. Gallinarum and S. Pollorum.<sup>23</sup> However, in the study, results showed the moderate to high degree of its correlation toward the standard conventional sero-agglutination. Nine of HRM profiles were unique to the single serotype, of each. One exception is the HRM\_3, which were belonged to five *Salmonella* serotypes. Higher variation of *Salmonella* serotypes tested in the current study, as well as analogous of nucleotide sequence of the three

PCR-targeted genes, *fljB*, *gyrB* and *ycfQ* are possibly played the role on the circumstance.

Although, HRM analysis could not discriminate the five of 14 *Salmonella* serotypes in the study. However, overlapping of the 95% confidence intervals of Simpson's diversity index suggests the discriminatory power of the two methods is not much different. Measured in Wallace coefficient, the value of HRM analysis to the conventional serotyping was 0.813. *Salmonella* strains which are found to show a similar HRM profile will have 81.3% chance of being grouped in a similar serotype when assessed by conventional serum agglutination method. Serotyping predictability by HRM analysis may be accepted for applied in field practice, in some serotypes.

## Conclusion

HRM analysis method was successfully introduced to *Salmonella* characterization. Dissociation curves profiles based on the polymorphism of three unique genes, *fljB*, *gyrB* and *ycfQ* could differentiate almost *Salmonella* serotypes in this study. Nevertheless, it can be currently revealed as an alternative approach for direct *Salmonella* serotyping, with the speedy and labor-saving procedure.

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## Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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