High resolution melting (HRM) analysis as a new tool for rapid identification of *Salmonella* enterica serovar Gallinarum biovars Pullorum and Gallinarum

Xingxing Ren, Ying Fu, Chenggang Xu, Zhou Feng, Miao Li, Lina Zhang, Jianmin Zhang , and Ming Liao

Key Laboratory of Veterinary Vaccine Innovation of the Ministry of Agriculture, Key Laboratory of Zoonosis Prevention and Control of Guangdong Province, PR China, College of Veterinary Medicine, South China Agricultural University, Guangzhou 510642, China

Abstract

- *Salmonella* enterica serovar Gallinarum biovars Pullorum and Gallinarum represent the most common causative agents of chicken salmonellosis, which result in high mortality and morbidity in throughout the world. It is difficult and laborious to discriminate these diseases based on biochemical or phenotypic methods. Herein, we report the development of a single nucleotide polymorphism (SNP) PCR-high resolution melt (PCR-HRM) assay for the detection and discrimination of both S. Pullorum and S. Gallinarun. The gene rfbS, which encodes a factor involved in the biosynthesis of ADP paratose in serogroup D of *Salmonella*, has been identified as robust genetic marker for the identification of S. Pullorum and S. Gallinarun based on polymorphisms at positions 237 and 598. Therefore, PCR-HRM analyses were used to characterize this gene.
- A total of 15 reference and 33 clinical isolates of *Salmonella* and related Gram-negative bacteria were detected using two sets of primers. Our PCR-HRM assay could distinguish S. Pullorum from S. Gallinarun and other strains using the primer pair SP-237F/237R. Similarly, S. Gallinarun could be distinguished from S. Pullorum and other strains using primer set SG-598F/598R. These two assays showed high specificity (100%) for both S. Pullorum and S. Gallinarun; the sensitivity of these two assays was at least 100-fold greater than that of the allele-specific PCR assay.
- This present study demonstrated that HRM analysis represents a potent, simple, and economic tool for the rapid, specific, and sensitive detection of S. Pullorum and S. Gallinarun. Our approach may also aid efforts for purification of Avian *Salmonella* disease.

Introduction

- Pullorum disease (PD) and fowl typhoid (FT) are 2 distinct septicaemic diseases that specifically affect avian species of major economic significance in many regions worldwide. *Salmonella* enterica serovar Gallinarum biovars Pullorum (S. Pullorum) and Gallinarum (S. Gallinarum) are the causative agents of these 2 diseases. S. Pullorum and S. Gallinarum belong to Kauffmann–White scheme serogroup D. Both and are indistinguishable that display ‘O’ antigens 1, 9, and 12, and exhibit high cross-reactivity with both each other and other serogroup D serovars, such as S. Enteritidis.
- High resolution melting (HRM) analysis represents a post-PCR technique that was developed for mutation scanning in nucleic acid sequences. It has been previously used to identify viruses, bacteria, nematodes, and fungi. Herein, To combat these pathogens, we developed a PCR-HRM curve assay to detect and discriminate S. Pullorum and S. Gallinarum based on SNPs in the rfbS gene at positions 237 and 598.

Methods

Sample collection and DNA extraction

Strain and serogroup	Coding	Source	Number	Tm (°C)	
				*rfbS*237	*rfbS*598
S. Pullorum	CVCC535	CVCC[1]	1	70.32	72.90
S. Gallinarum	CICC21510	CICC[2]	1	69.71	72.41
S. Enteritidis	ATCC13076	ATCC[3]	1	69.71	72.90
Typhimurium	ATCC14028	ATCC	1	NA[4]	NA
S. Indiana	ATCC51959	ATCC	1	NA	NA
Hadar	ATCC51956	ATCC	1	NA	NA
S. Agona	ATCC51957	ATCC	1	NA	NA
S. Weltevreden	ATCC BAA-2568	ATCC	1	NA	NA
S. Meleagridis	CICC21511	CICC	1	NA	NA
S. Thompson	ATCC8391	ATCC	1	NA	NA
S. Senftenberg	ATCC43845	ATCC	1	NA	NA
S. Stanle	ATCC7308	ATCC	1	NA	NA
S. Infantis	ATCC BAA-1675	ATCC	1	NA	NA
Escherichia coli	ATCC25922	ATCC	1	NA	NA
Pasteurella multocida	ATCC12948	ATCC	1	NA	NA
S. Pullorum	SP-gd01–SP-gd22	Field sample	22	70.32 ± 0.04	72.90 ± 0.03
S. Gallinarum	SG-gd01–SG-gd07	Field sample	7	69.71 ± 0.04	72.41 ± 0.02
S. Enteritidis	SE-gd01–SE-gd04	Field sample	4	69.71 ± 0.04	72.90 ± 0.03

[1] CVCC, China Veterinary Culture Collection Center.
[2] CICC, China Center of Industrial Culture Collection.
[3] ATCC, American Type Culture Collection.
[4] NA, not available.

Primer design

Primer	Sequence (5′ à3′)	size	Interpretation
SP-237FSP-237R	AAAGCAATATTCTTATGCCTACAC AATTTATGAATACTGCATC	72 bp	HRM primer can differentiate *S.* Pullorum
SG-598FSG-598R	TGTTAATAATTTCCCCAAAGTCTC TACATATTCACG	79 bp	HRM primer can differentiate *S.* Gallinarum
rfbS-FrfbS-R	ACATACTGTGATTGGCTTAGCATT GGCTCTTTCTTTGA	661 bp	Pre-amplification and sequencing primer

PCR-HRM Analysis

Reagent	Dose
PremixEx-Taq mixture	5.0μL
rfbS-F	0.5μL
rfbS-R	0.5μL
Template DNA	1.0μL
EvaGreen fluorescent dye	1.0mL
ddH2O	2.0μL
Total	10μL

- Reactions were carried out using a Corbett Rotor-Gene Q™ (Qiagen) with an initial denaturation step of 95°C for 5 min, 40 cycles of 95°C for 20 s, 54°C for 20 s, and 72°C for 20 s. After amplification, HRM analysis was performed between 60°C and 85°C at a rate of 0.3°C.

Specificity and Sensitivity Testing

- Conventional amplification of the rfbS gene (primer rfbS-F/R) of standard bacteria strains S. Pullorum and S. Gallinarun was performed.
- Amplified rfbS genes were cloned into pMD19-T vectors (TaKaRa).
- pMD19-T vectors were sequenced and validated.
- Positive plasmids were diluted in 10-fold serial dilutions with ddH2O and the minimum copy number was detected in the optimised PCR-HRM reaction.

Results

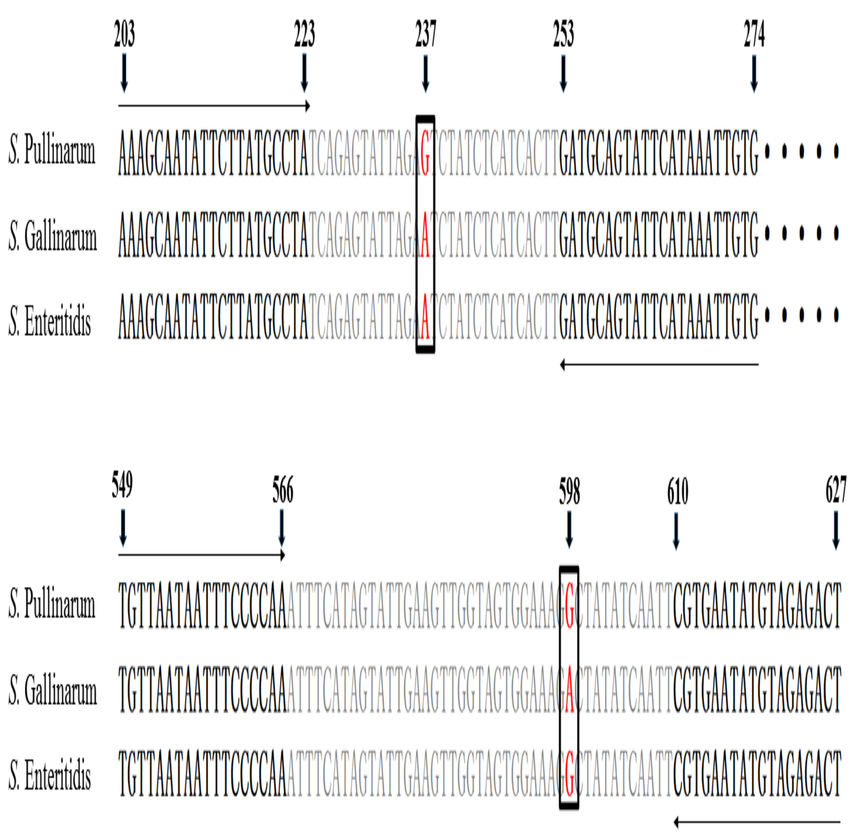

Fig 1: The two amplicon fragments amplified by primer SP-237F/237R and primer SG-598F/598R in this study. The SNPs are indicated by the box.

Results

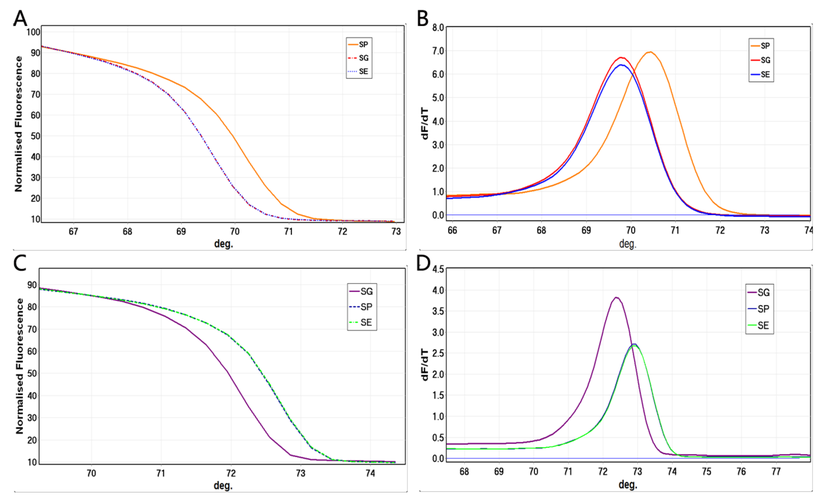

Fig 2: Conventional and normalized melt curves analysis of PCR products from reference strains amplified by primer SP-237F/237R (A and B). Conventional and normalized melt curves analysis of PCR products from reference strains amplified by primer SG-598F/598R (C and D).

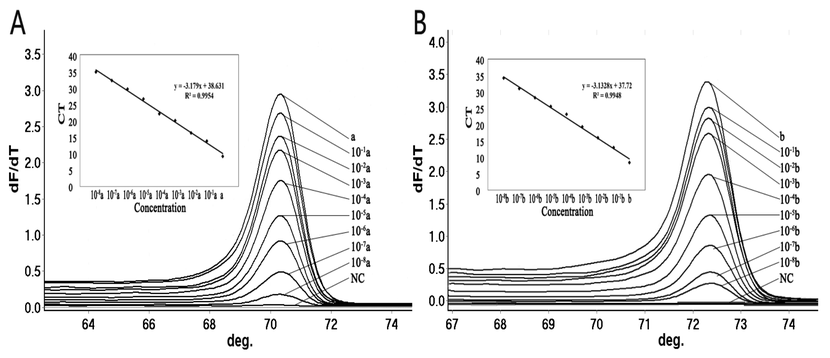

Fig 3: The sensitivity of the PCR-HRM based on EvaGreen for the detection S. Pullorum and S. Gallinarum. (A) Normalized melt curve analysis in serial dilution of S. Pullorum DNA (amplicon “a” is the concentration of PMD19T-SP) and a standard curve of S. Pullorum performed in a linear graph with R2 0.995. (B) Normalized melt curve analysis in serial dilution of S. Gallinarum DNA (amplicon “b” is the concentration of PMD19T-SG) and a standard cure of S. Gallinarum performed in a linear graph with R2 0.995.

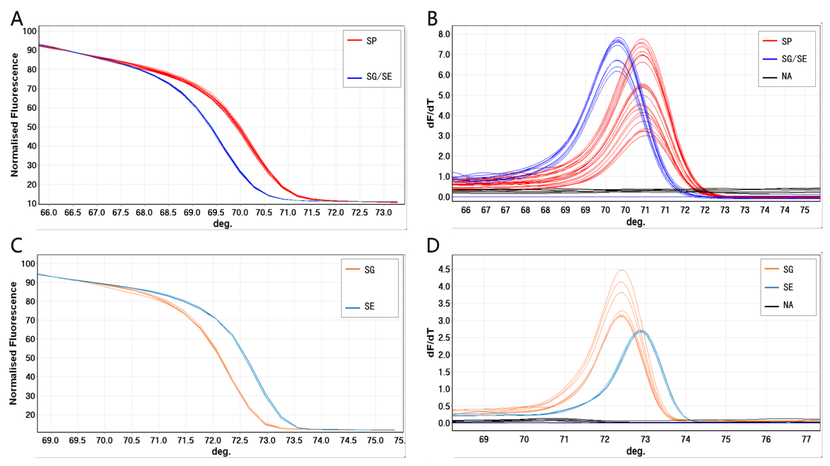

Fig 4: Conventional and normalized melt curves analysis of PCR products from field strains amplified by primer SP-237F/237R (A and B). Conventional and normalized melt curves analysis of PCR products from field strains amplified by primer SG-598F/598R (C and D).

Conclusions

- This represents the first report of the successful application of PCR-HRM technology to distinguish S. Pullorum, S. Gallinarum, and other strains. Each assay was shown to be sufficiently sensitive and specific to allow the melting profiles to distinguish different characteristics of specific mutations.
- Our findings suggest that PCR-HRM analysis represents a single-step, closed-tube, and economical procedure for the rapid, specific, and sensitive detection of both S. Pullorum and S. Gallinarum.

References

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