Global Veterinaria 13 (2): 209-214, 2014
ISSN 1992-6197

DOI: 10.5829/idosi.gv.2014.13.02.84103

Application of SYBR Green Real-Time PCR to Detect *Salmonella enterica* Serotype Enteritidis, Typhimurium and Kentucky in Poultry

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Abstract: The study highlights that, SYBR Green real-time PCR test is a rapid, reproducible and robust method for detecting *Salmonella enteritidis*, *Salmonella Typhimurium* and *Salmonella Kentucky* in poultry samples using primers SEFA-2 and SEFA-4 for *S. enteritidis*, fli 15 and tym for *S. Typhimurium* and *S. kentucky*, respectively. The specificity of the reaction was confirmed by the melting temperature (Tm), which was consistently specific for the amplicon obtained; the mean peak Tm obtained with curves specific for serotype Enteritidis was 83.03°C while it was 83.99°C and 84.01°C for *S. Kentucky* and *S. Typhimurium*, respectively. The results of this study demonstrate that the SYBR Green I real-time PCR constitutes an effective and easy-to-perform method for detecting *S. enteritidis*, *S. Typhimurium* and *S. kentucky* in poultry samples.

Key words: Polymerase Chain Reaction · Nucleic Acid Amplification–Isolation · Serovars · Salmonella

INTRODUCTION

Salmonella is a genus of Gram-negative rod-shaped bacteria of the family Enterobacteriaceae causing a wide range of human diseases [1]. Salmonella species produce many disease forms that range from a mild enteritis to severe systematic infection in a variety of animal hosts [2]. In addition, salmonellosis is still one of the major global causes of gastroenteritis in humans and animals [3]. More than 2500 serotypes of salmonellae have been described, mostly belonging to the species *Salmonella enterica* [4]. Some of these serotypes, such as *S.* Typhimurium and *S. enteritidis*, can infect humans while other serotypes are host-specific, infecting a single species and generally causing severe typhoid-like symptoms sometimes leading to death (for instance, *S. infantis* and *S. kentucky* in poultry). These serotypes can be responsible for disease outbreaks leading to severe economic losses [5]. European Food Safety Authority accounted *S. enteritidis* and *S.* Typhimurium for most zoonotic salmonellosis associated with food of animal origin [6]. Therefore, the prevalence of salmonella species in food production animal herds constitutes a challenge for safe food production [7].

Salmonella spp. in foods can be detected by various methods such as conventional bacteriological culture, serological assays, polymerase chain reaction (PCR) and more recently, real-time PCR methods [8]. Real-time PCR system reduces the detection time, however, its application requires the availability of primers and probes that must be selected according to very rigid conditions, which cannot always be easily applied. The use of the double-stranded DNA (dsDNA) binding dye SYBR Green for the detection of PCR products has overcome this limitation by allowing real-time PCR to be applied without the need for probes linked to fluorescent molecules [9,10,11]. In the absence of probes, the specificity of the reaction is determined by the melting temperature (Tm) of the amplicon obtained, defined as the temperature at which 50% of the DNA amplicon is in a double-stranded configuration. The Tm depends on

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various factors, including the concentration of the dsDNA, the amplicon length and the nucleotide sequence [12]. Salmonella infection in humans can be reduced through upgrading methods of detection [13].

Significance and Impact of the Study: SYBR Green RT-PCR is a powerful tool for rapid and accurate salmonella monitoring in poultry companies, together with standard bacteriological testing.

MATERIALS AND METHODS

Sample Collection: A total of 1200 internal organs of diseased and apparent healthy layer chicken (liver, gall bladder, spleen, intestine and ovary) (n=150), internal organs of diseased and apparent healthy broiler chicken (liver, gall bladder, spleen and intestine) (n=100), Specific pathogen free (S.P.F) and commercial (native, white, brown) eggs (n=600), Feed stuff (n=200) and litter samples (n=150) were either submitted to the Animal Health Research Institute, Dokki, Giza or brought from different markets or different poultry farms in Cairo, Giza and Fayoum governorates under aseptic conditions in ice box with ice packs (at 4°C) and transferred to the laboratory.

Isolation and Identification: The method for detection, isolation and identification of Salmonella spp. was done according to ISO 6579]14[method.

Serological Confirmation: Diagnostic monovalent, polyvalent I, II, III and monovalent Salmonella O and H (phase 1 and phase 2) antisera. (Denka Seiken co., LTD) & (Pro–lab diagnostic, U.K). The positive Salmonella colonies biochemically were confirmed by monovalent antisera by slide agglutination test.

Materials

Materials Used for Extraction of DNA:

- QIAamp DNA Mini Kit (QIAGEN/Catalogue no.51304)

The QIAamp DNA Mini Kit provides silica-membrane-based nucleic acid purification from different types of samples. The spin-column procedure does not require mechanical homogenization, so total hands-on preparation time is only 20 minutes.

- Ethanol 96% (Applichem)

Equipment and Apparatuses Used for Extraction of DNA:

- Epindorf tubes 1.5 ml capacity
- PCR tubes 0.2 ml capacity
- Monochannel micropipettes 10-100 μl, 100-1000 μl (Biohit)
- Sterile filter tips (100 μl, 1000 μl) capacity
- Centrifuge (Sigma Sartorius)
- (-20°C) Freezer (Toshiba)
- Type II-A biosafety cabinet (Thermo)
- Thermoblock (Biometra)

Materials Used for Mastermix Preparation for SYBR Green Real-Time PCR:

- Quanti Tect SYBR Green PCR kit (QIAGEN/Catalogue no.204141)

Equipment and Apparatuses Used for Real-Time PCR:

- Real-time PCR machine (Stratagene MX3005P)

The MX3005P QPCR system is a fully integrated QPCR detection and data analysis system. The system design combines the state-of-the-art thermal cycler, an advanced optical system with quartz-tungsten halogen lamb and a single photomultiplier tube (PMT) and the most powerful analysis software available. Five selected filter sets are provided with the system and the scanning optics design delivers optimal separation between the dyes and between the samples.

- Unichannel micropipettes (100-1000), (2-20), (0.5-10) and (20-200) µl (Biohit)
- Filter tips of different sizes
- Optical tubes (0.2 ml) (Applied biosystem)
- Optical caps (Applied biosystem)

Methods

Extraction of DNA:

- QIAamp DNA Mini Kit (QIAGEN/Catalogue no. 51304)

Preparation of PCR Master Mix According to QuantiTect SYBR Green PCR Kit

Cycling Conditions for SYBR Green Real-Time PCR:

- Cycling conditions for SYBR Green real-time PCR of *S. enteritidis* (according to QuantiTect SYBR Green PCR kit and Akbarmehr *et al.* [16]).

Table 1: Sequence of Oligonucleotides (Source: Metabion/Germany)

Agent	Primer	Target gene	Primer sequence (5'-3')	Length of amplified product
*S. Typhimutium*and	*fli 15*	*fli*C	CGGTGTTGCCCAGGTTGGTAAT	559 bp [15].
S. Kentucky	*tym*		ACTCTTGCTGGCGGTGCGACTT	
S. Enteritidis	SEFA2	*sefA*	GCAGCGGTTACTATTGCAGC	310 bp [16].
	SEFA4		TGTGACAGGGACATTTAGCG	

Table 2: Components of PCR Master Mix

Component	Volume/reaction
2x QuantiTect SYBR Green PCR Master Mix	12.5 µl
Forward primer (50 pmol)	0.5 µl
Reverse primer (50 pmol)	0.5 µl
RNase Free Water	4.5 µl
Template DNA	7 µl
Total	25 µl

Table 3: Cycling conditions for SYBR Green real-time PCR of *S. enteritidis*

Stage	Temperature	Time	Cycles
Primary denaturation	94°C	5 min.	1
Amplification			
a) Secondary denaturation	94°C	30 sec.	40
b) Annealing	52°C	30 sec. (optics on)	
C) Extension	72°C	30 sec.	
Dissociation curve			
a) Secondary denaturation	95°C	1 min.	1
b) Annealing	52°C	1 min. (optics on till final denaturation)	
a) Final denaturation	95°C	30 sec.	

Table 4: Cycling conditions for SYBR Green real-time PCR of *S.* Typhimutium *and S. kentucky*

Stage	Temperature	Time	Cycles
Primary denaturation	94°C	10 min.	1
Amplification			
a) Secondary denaturation	94°C	45 sec.	40
b) Annealing	56°C	45 sec. (optics on)	
C) Extension	72°C	45 sec.	
Dissociation curve			
a) Secondary denaturation	95°C	1 min.	1
b) Annealing	56°C	1 min. (optics on till final denaturation)	
a) Final denaturation	95°C	30 sec.	

Cycling conditions for SYBR Green real-time PCR of *S. Typhimurium* and *S. kentucky* (according to QuantiTect SYBR Green PCR kit and Soumet *et al.* [15]).

RESULTS

In this study, 64 samples (5.33%) out a total of 1200 internal organs of diseased and apparent healthy layer chicken (liver, gall bladder, spleen, intestine and ovary) (n=150), internal organs of diseased and apparent healthy broiler chicken (liver, gall bladder, spleen and intestine) (n=100). Samples included Specific Pathogen Free (S.P.F) and commercial (native, white, brown) eggs (n=600), feed stuff (n=200) and litter samples (n=150) in which *Salmonella* spp. was identified serologically to: *S. enteritidis, S.* Typhimurium, *S. kentucky, S. arizonae, S. hydra, S. anatum, S. paratyphi* A, *S. agona, S. bloomsburg, S. derby, S. rubislaw, S. senftenberg*, *S. virchow*, *S. cerro* in incidences of: 10.9%, 32.8%, 10.9%, 7.8%, 3.1%, 1.6%, 1.6%, 6.3%, 1.6%, 3.1%, 4.6%, 1.6%, 6.3% and 7.8%, respectively.

Detection of *Salmonella enteritidis*, *Salmonella* Typhimurium and *Salmonella kentucky* in poultry samples were performed using primers SEFA-2 and SEFA-4 for S. *enteritidis*, fli 15 and tym for S. Typhimurium and S. *kentucky*, respectively. The specificity of the reaction was confirmed by the Tm, which was consistently specific for the amplicon obtained; the mean peak Tm obtained with curves specific for *S. enteritidis* was 83.03°C (Fig. 1) while, it was 83.99°C and 84.01°C for *S. kentucky* and

Table 5: Dissociation results for *S. enteritidis*

Well	Well Name	Dye	Tm Product 1 (-R'(T))
A1	Neg. *S.* Typhimurium	SYBR	78.58
B1	*S. enteritidis*	SYBR	83.03
C1	Pos. *S. enteritidis*	SYBR	83.50

Table 6: Dissociation results for *S.* Typhimurium and *S. Kentucky*

Well	Well Name	Dye	Tm Product 1 (-R'(T))
A1	Neg. *S. enteritidis*	SYBR	81.92
B1	S. *kentucky*	SYBR	83.99
C1	S. Typhimurium	SYBR	84.01
D1	Pos. *S.* Typhimurium	SYBR	84.04

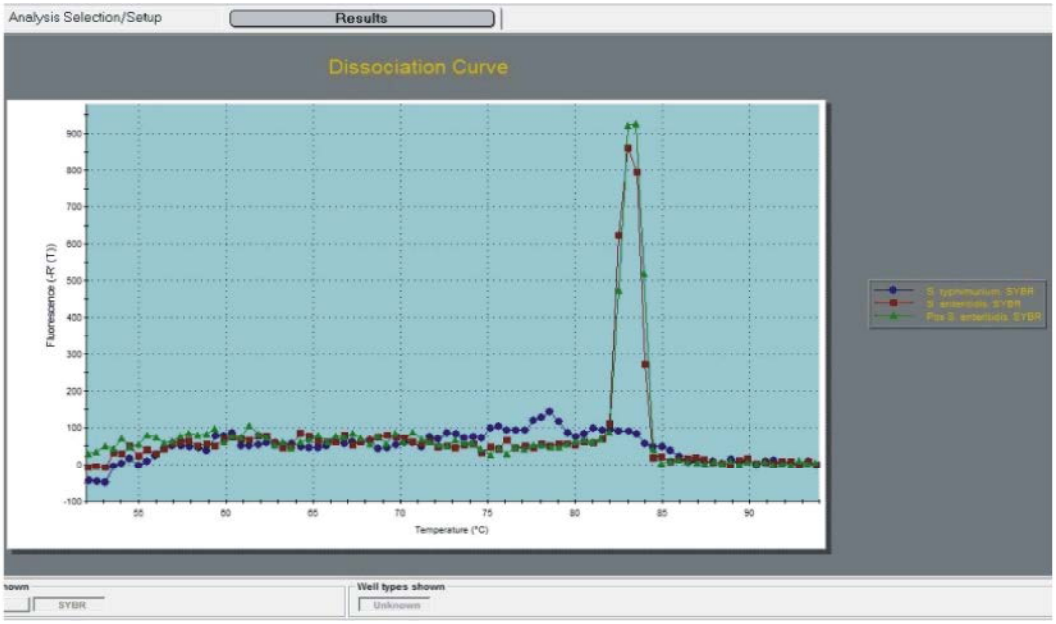

Fig. 1: Melting curve of *S. enteritidis* after 40 cycles

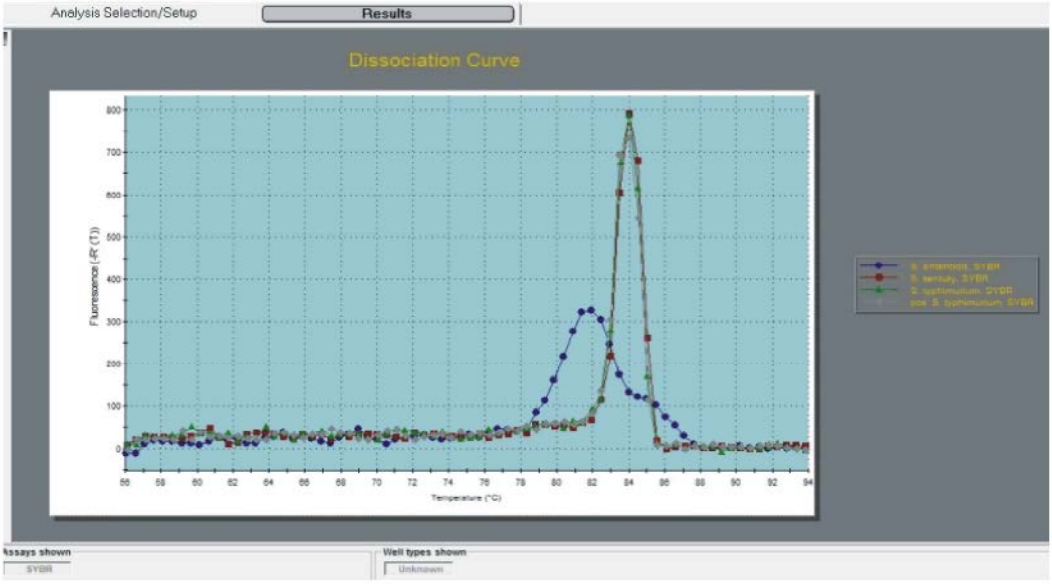

Fig. 2: Melting curve of *S. Typhimurium* and *S. kentucky* after 40 cycles

S. Typhimurium, respectively (Fig. 2). The negative controls and the samples contaminated with serotypes other than serotype enteritidis did not show peaks in the Tm when they were subjected to 35 cycles of amplification whereas they did show some peaks (for example, *S.* Typhimurium) when they were subjected to 40 cycles.

DISCUSSION

In this study SYBR Green real-time PCR test was made for developing a rapid, reproducible and robust method for detecting *Salmonella enteritidis*, *Salmonella Typhimurium* and *Salmonella kentucky* in poultry samples using primers SEFA-2 and SEFA-4 (310 bp) for *S. enteritidis* and fli 15 and tym (559 bp) for *S.* Typhimurium and *S. kentucky*, respectively. The specificity of the reaction was confirmed by the melting temperature (Tm), which was consistently specific for the amplicon obtained. The mean peak (Tm) obtained with curves specific for serotype enteritidis was 83.03°C (Fig. 1) while it was 83.99°C and 84.01°C for *S. kentucky* and *S.* Typhimurium, respectively (Fig. 2). The specificity of the reaction was confirmed by the determination of the Tm, which was consistently specific for the amplicon obtained. Determining the Tm has the marked advantage of eliminating the phase of electrophoresis, which is time-consuming, carries the risk of laboratory contamination with nucleic acid due to post-PCR manipulation [17] and requires the use of ethidium bromide, which, being a potent mutagenic agent, is not suitable for routine use [18]. However, variations of more than 1°C can occur in the minimum and maximum Tms, as have also been reported by other studies [19, 20]. These variations are in part due to a non-homogeneous distribution of the temperature in the thermocycler. The standard curve showed a strict inverse correlation between the CT and the concentration of salmonella in the samples and the limit of sensitivity of the method was less than 10^3 CFU/ml. Based on the CT values found in the samples containing serotype enteritidis under our experimental conditions, the concentration of the microorganism in the pre-enrichment culture after 18 to 20 hours of incubation at 37°C was always greater than 10^8 CFU/ml.

It can be concluded that the incubation period of the pre-enrichment medium can be reduced, thus shortening the time required for this method while maintaining its effectiveness and rendering it particularly useful for determining the effectiveness of the hazard analysis and critical point systems used in the food industry to reduce the numbers of pathogenic germs, which agrees with the results of Hofstra *et al*. [21].

ACKNOWLEDGEMENTS

Thanks are dedicated to Microbiology Department, Faculty of Veterinary Medicine, Cairo University, Egypt.

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