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Use of Real-Time PCR and High-Resolution Melting Analysis for Detection and Discrimination of *Salmonella typhimurium* and *Salmonella enteritidis* in Contaminated Raw-Egg Samples

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ABSTRACT: In order to evaluate the efficacy of high-resolution melting (HRM) analysis in the detection and discrimination of *S. typhimurium* and *S. enteritidis*, this study was conducted on 30 samples of raw eggs, where 14 treatments were employed: 4 groups of samples contaminated with *Salmonella typhimurium*, 4 with *S. enteritidis*, 4 with a mixture of both, as well as 2 control groups. DNA was extracted from the contaminated samples and used for preparation of a target DNA sequence using an *invA* gene-specific primer pair. RT-PCR identified and detected *Salmonella* in the contaminated egg samples within less than 24 hours, and HRM analysis proved that this method is suitable for the discrimination of the *S. enteritidis* and *S. typhimurium* species. The sensitivity of this method was equal to 200 *Salmonella* cells per microliter, and HRM analysis could distinguish *S. enteritidis* and *S. typhimurium* and their combination with high consistency ($R^2 > .993$).

Keywords: *HRM, Melting Curve, Real Time-PCR, Salmonella enteritidis, Salmonella typhimurium*.

Introduction

Salmonella is a gram-negative, facultative, anaerobic, rod-shaped bacterium belonging to the *Enterobacteriaceae* family (Janda and Abbot, 2006). This bacterium is one of the chief reasons for foodborne illnesses worldwide (Anvar *et al*., 2019) *Salmonella enteritidis* is one of the main causative agents of salmonellosis in the world. According to research, *S. enteritidis* and *S. typhimurium* can cause infection in the hen genitals and contaminate an egg while it is being formed. This is why *S. enteritidis* usually contaminates the hen after the hen hatches the eggs (Malorny *et al*., 2004). *Salmonella typhimurium* is also one of the causes of typhoid fever, and meat supplies are known as its main transmission medium (Vellai. And Vida, 1999). *Salmonella* has some specific genes named *int*, *spr*, *inv*, *fliC*, and *invA* that play a role in the pathogenicity, and because of these genes, *Salmonella* can reach deeper parts of the colon (Gbassi and Vandamme, 2012).

Nowadays, fast detection methods for diagnosis and prevention of foodborne illnesses are essential in case of the emergence and spread of these illnesses.

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Hence, for acceleration of the analysis and detection of pathogens in food products, the PCR method and more recently the RT-PCR method have been used (Hein *et al*., 2006).

The use of HRM as one of the newest advances in the molecular detection of bacteria is helpful and is in fact a method applied after PCR and recognizes the changes in nucleotide sequences using the analysis of amplicon melting curves (Souza *et al*., 2015).

Many scientists have carried out studies on the detection and discrimination of *Salmonella* species in food, for example, De Medici (2003) tried to evaluate different methods of DNA analysis involving real-time PCR and SYBR Green I for detecting *Salmonella enterica* in poultry meat samples, which were manually contaminated with *Salmonella* or were acquired on the market (De Medici *et al*., 2003). Moreover, Singh *et al*. (2014) conducted a study and tried to detect *Salmonella* species resistant to an antibiotic using multiplex Real Time-PCR with two primers specific to 41 species of *Salmonella,* and 8 samples were manually contaminated with *Salmonella* (Singh and Mustapha, 2014).

In the present study its tried to compare and evaluate the advantages of Real Time PCR and HRM analysis in terms of speed and sensitivity of detection of this bacterial genus in different samples including egg samples.

Materials and Methods

- *Preparation of the eggs and the samples*

30 fresh raw egg samples supplied by protein shopping centers of District 2 in Tehran Province with random sampling were analyzed in 14 treatment groups (4 groups for studying S. typhimurium, 4 groups for S. enteritidis, and 4 groups for the mixture of these two bacteria, along with 2 control groups). Initially, to ensure that the contamination of egg shells does not transfer to their content after breaking of the shells, the shells were sterilized with 70% ethanol, and the egg contents including yolk and Albumen were poured into 250-ml sterile glass vessels with a cover and were homogenized.

- *Testing for the absence of Salmonella in samples*

Tracking of *Salmonella* in food products using the De Esmdt (1986) method was performed using microbial culture at several stages (De SMEDT et al., 1986). After the pre-enrichment of the egg samples, which was carried out in the bottles containing nonselective broth culture (Phosphate Buffer Agar) inside a 37°C incubator for 18 to 20 hours, enrichment was performed on the samples by taking 1 ml of the pre-enriched sample with a sampler and transferring it to a 10 ml culture of Rappaport Vassiliadis Broth. Each sample was then placed in a 37°C incubator and incubated for 24 hours. A loop ful of the Rappaport liquid culture was taken and seeded on the MacConkey agar medium in proximity of a flame for sterility. The plates were then incubated at 37°C for 24 hours. After the analysis of the culture medium, no changes were observed, indicating the lack of *Salmonella* in the culture.

- *Preparation and enrichment stage*

S. typhimurium (ATCC14028) and *S. enteritidis* (13341 ATCC) were purchased (from Iran Organization of Scientific & Industrial Research as live cultures). After the culture grew, the target bacteria were taken with a sterile loop and inoculated into the lab tubes containing 9 ml of 1/500 diluted nutrient broth separately and after homogenization, 10^8 CFU/ml concentration was attained (Ahari et al., 2020). In order to confirm the obtained optical density, besides a visual comparison with McFarland Standards, a spectrophotometer at 420-nm wavelength was used. Different final concentrations from 10^1 to 10^7 CFU/ml were

prepared for *S. enteritidis*, *S. typhimurium*, and the mixture of the two bacteria. Then, the 10^7 CFU/ml injected sample was also prepared. This process continued for all concentrations up to 10^0 dilutions (the above-mentioned process was performed for each bacterium and their mixture). To count the bacterial cells, the suspensions of 10^1, 10^4, and 10^7 CFU/ml were used randomly. The number of bacterial cells on the plate was calculated by multiplying the bacteria placed on the plate by 10 times the dilution and by the number of counted colonies.

- *Extraction of sample DNA*

The samples were brought to the ambient temperature and homogenized, then 100 microliters of samples which contained 10^5 to 10^8 bacterial cells were transferred to 400-µl tubes. Subsequently, 20 µl of proteinase K and then 180 µl of lysis buffer were added to each tube, and they were incubated for 1 to 3 hours and centrifuged for 10 seconds in order for the cell wall to be slippery. Afterwards, 20 µl of binding buffer was then added to the tubes and vortexing was done for 10 seconds. To finish the late stage of the tube processing, the samples were incubated for 10 minutes at 70°C. After that, 200 µl of 100% ethanol was added into the tubes, and all the tubes were vortexed for 10 seconds and centrifuged for another 10 seconds. For all the samples, 2-ml microtubes were set up with a DNA-binding column in them. Finally, by pipetting, each sample was transferred to the DNA-binding column and centrifuged for 1 minute in 8000 rpm. Next, 500 µl of washing buffer 1 was added into the columns, and they were centrifuged for 1 minute at 8000 rpm. The tubes under the columns were thrown away, and the column was transferred to a fresh 2-ml microtube. After that, 500 µl of washing buffer 2 was added into the columns, and they were centrifuged for 3 minutes at 14000 rpm. The tubes under the columns were thrown away, and the column was transferred to a 200-µl microtube and 100 µl of the elution buffer was added into each column, and the latter was centrifuged for 1 minute at 13000 rpm. Finally, the purified DNA was stored at –20°C. At the next stage of the DNA extraction from pure bacteria, the boiling method was used where 3 colonies were taken from each culture and transferred to the microtubes containing distilled water. The microtubes were placed in a water bath at 100°C, with incubation for 15 minutes, and then centrifuged for 5 minutes at 1300 rpm. Next, 300 µl of the resulting solution was transferred to the new tube and centrifuged again. At last, 100 µl of purified DNA was prepared.

- *Real Time-PCR*

These reactions were carried out based on the experiments designed by Bratchikov and Mauriac (2011), where a reaction mixture (shown in Table 1) was used to amplify the extracted DNA (Bratchikov and Mauricas, 2011). The aforementioned mixtures were brought to a final volume of 25 µl and subjected to RT-PCR. Simultaneously, 2 µl of distilled water along with the PCR mixture in a tube labeled as BLANK were included in the PCR assay along with the other samples (Bratchikov and Mauricas, 2011). To amplify the intended gene, initial denaturation was commenced at 95°C for 4 minutes and afterwards, ~35 cycles at 95°C for 35 seconds, 50°C for 30 seconds (primer annealing), and ~72°C for 20 seconds (elongation) were run. Next, the fluorescent signals from each sample were recorded for each cycle by the software. We used a pair of primers specific for gene *invA*, which is related to the genome region of type II released triphosphate of adenosine-5′ connection protein. This primer pair was employed for the detection of both *S. enteritidis* and *S.* typhimurium:

5′-CCGGTTCGCCAGGATACAAGCCTG-3′
5′-GCCCAGAGCGCGCAATGGCGTCAG-3′

- *Analysis of melting curves*

During the analysis of melting curve performed with high resolution, the temperature increased from 84°C to 92°C in .2°C steps per second. The constant decrease of fluorescent signals in this system before and after this stage was used to draw a melting curve based on the initial florescence curve. This curve was ultimately subjected to HRM analysis for quantitative PCR after normalization of the curve.

Results and Discussion

In order to count the bacterial cells in egg samples, the three concentrations of 10^1, 10^4, and 10^7 CFU/ml were randomly used and the plates were incubated for 24 to 48 hours at 37°C; counting was performed according to the formula mentioned in the previous section. To count the microbial load of 10^7 CFU/ml, the dilutions of 10^{-3} to 10^{-1} were used, which were more suitable than the 10^{-6} dilution and better for counting because of the optimal concentration of the colonies. Ultimately, the number of bacterial cells on the plate was found to be 1.7×10^8. In the rest of counting assays, the microbial load of 10^4 CFU/ml, at 10^{-3} to 10^{-1} dilutions was used where the 10^{-3} dilution was more convenient for counting, and the number of bacterial cells equaled 2.3×10^5 per plate. In addition, for counting the microbial load of 10^1 CFU/ml, a 10^{-1} dilution was used. In this plate, there were 1.5×10^3 bacterial cells, indicating that the growth of bacterial cell number was similar among different plates with the same bacterial load. Additionally, the microbial load of each count increased one log due to the time interval during the process.

- *Optimization of PCR conditions*

DNA (2 µl) from *S. enteritidis* and *S. typhimurium* (from eggs contaminated at 10^7 CFU/ml), and from pure *S. enteritidis* and *S.* typhimurium bacteria as the positive control was added to 23 µl of a PCR mix. Besides, 5 µl of distilled water was added to the 23 µl of the PCR mix as a blank sample. The RT-PCR assay was performed using the following pair of primers from the study by Bratchikov and Mauricas (2011) for *CR2* CRISPR gene (Bratchikov and Mauricas, 2011).

CRF: 5′-AACGCCATGGCCTTCTCCTG-3′
CRR: 5′-CAAAATCAGYAAATTAGCTGTTC-3′

The six reactions shown in the following table 2 lasted for 4 hours in the block of the PCR cycler and were conducted on Rotor Gene 2.0.2.4.

The significance ranges of 90% were used, and the temperature for area 1 of normalization was between 86.78°C and 87.28°C, and for area 2 of normalization was between 93.03°C and 93.7°C. In this table, a total of three steps (denaturation, annealing,

Table 1. Materials used in mixed PCR

No.	Materials used	Required amount
1	PCR buffer	2.5 microliters
2	MgCl2	1 microliters
3	dNTP	2 microliters
4	Forward primer	0.5 microliters
5	Reverse primer	0.5 microliters
6	DNA Template	2 .5 microliters
7	Taq DNA polymerase	2.5 microliters
8	D.D.W	16.5 microliters
Total	25.5 microliters	Total volume of sample for PCR

Table 2. Temperature schedule of Real Time-PCR

No.	PCR Sections	Temperature	Time
1	Primary denaturation	95°C	4 min
2	Denaturation	95°C	20 s
3	Annealing	58°C	1 min
4	Elongation	72°C	2 min
5	Last elongation	72°C	5 min
6	Pre-HRM incubation	50°C	1 min
7	HRM region	80-95°C	

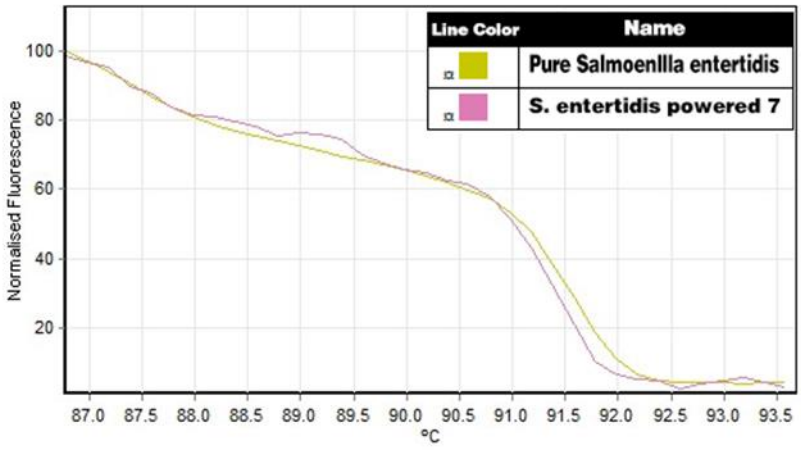

Fig. 1. The normalized graph in the experiment's optimization phase

and elongation) were performed for 35 cycles.

Regarding the normalized curve in Figure 1, our assay could detect only pure *S. enteritidis* bacteria and *S. enteritidis* in the contaminated egg samples, but could not detect *S. typhimurium*, either pure or injected into the egg.

- *Qualitative analysis of RT-PCR by HRM for detection of bacteria in the samples*

As in the previous section, 2 µl of the DNA from samples contaminated with *S. enteritidis* or *S.* typhimurium, as well as their mixture at 10^5 to 10^8 CFU/ml, or the DNA of pure bacteria was added to 23 µl of a PCR mix. At the optimization stage of the REAL TIME-PCR test results, the following primer pair was used for gene *invA*, and the 130-bp DNA fragment from the bacteria was amplified for the detection of both bacterial species.

5′-CCGGTTCGCCAGGATACAAGCCTG-3′
5′-GCCCAGAGCGCGCAATGGCGTCAG-3′

For the purpose of detection and discrimination of the *Salmonella* species, the following thermal cycling conditions (Table 3) were used.

Figure 2 shows the amplification of the target genome region during the PCR cycles in the presence of a saturated level of dye. The colors show that all of them have been targeted at this stage. From the 18 cycles, the ascending trend in all assays were observed and the final stage indicates the presence of all target treatments.

As demonstrated in Figure 3, panel A, the melting curve profiles, with a specific melting temperature (T_m) apex were

obtained for each bacterium at certain concentrations. According to this result, 2 different profiles were drawn for the bacterial assays in the picture, showing that the amplification in the PCR assay for *Salmonella* added to eggs yielded the average melting temperature of 86.86°C ± 0.08°C and for the two assays of pure *Salmonella*, the melting temperature was 90.42°C ± 1.06°C. Then, according to Figure 3 panel A, the melting temperatures were normalized in all the assays, and the method could detect pure *S. enteritidis*, *S. typhimurium*, or the mixture of the two pure bacteria.

Table 3. Temperature schedule in optimization section

	PCR Sections	Temperature	Time
1	Primary denaturation	95°C	4 min
2	Denaturation	95°C	5 s
3	Annealing	50°C	30 s
4	Elongation	72°C	20 s
5	Last elongation	72°C	2 min
6	Pre-HRM incubation	50°C	1 min
7	HRM region	84-92°C	

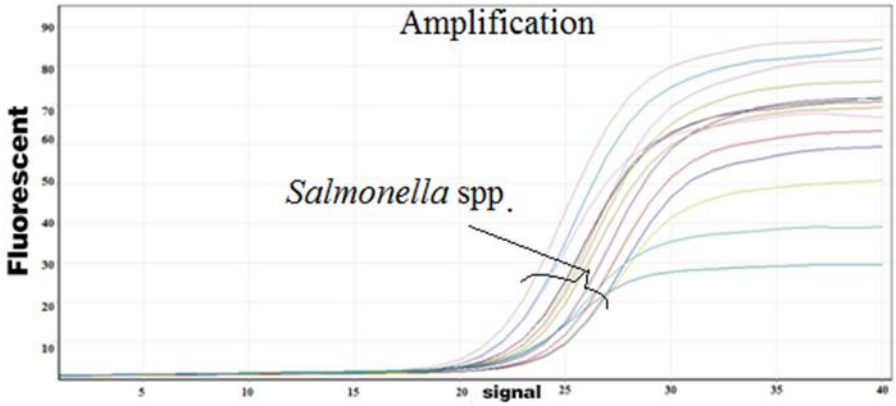

Fig. 2. The increase in target sequence copy numbers in cycles of different temperatures for different numbers of bacteria and at different initial concentrations of DNA

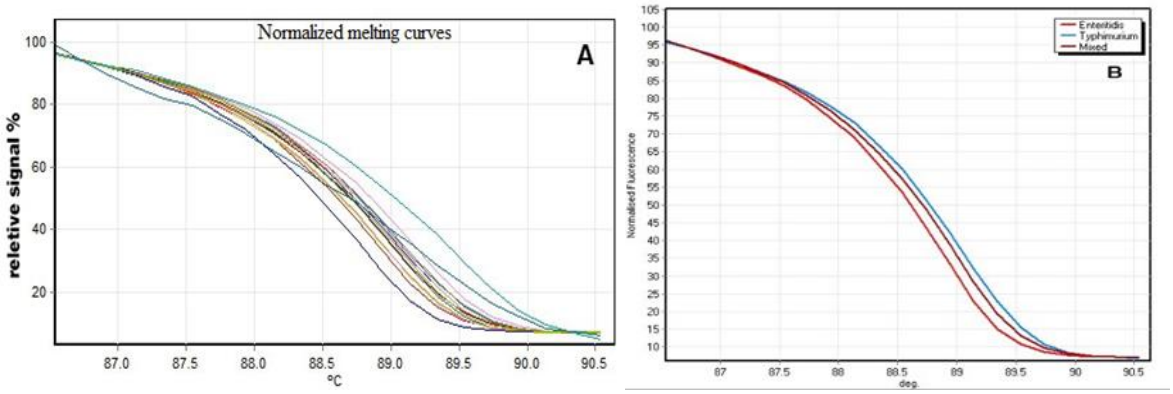

Fig. 3. Panel A shows the HRM results for 15 normalized melting curves, and panel B shows the HRM results for 2 normalized melting curves for pure

As indicated in Figure 3, the curves related to each assay are recognizable and distinguishable, indicating high specificity of REAL TIME-PCR. After the normalization of different assay graphs, as seen in Figure 3 panel B, 3 distinct curves that represent *S. typhimurium*, *S. enteritidis*, and the mixture of these two bacteria, respectively, could be observed.

According to the obtained results, the regression line equation was derived. Accordingly, it seems that the relation between the threshold cycle (C_T) and the concentration of *S. enteritidis* and *S. typhimurium* cells was reverse in the samples being tested. Thus, with each half unit decrease in the bacterial concentration, ~15 units are added to the threshold cycle. The regression line equation analysis was conducted here to identify a possible correlation between target *Salmonella* concentration in this study and the threshold cycle. In this analysis, the correlation coefficient was found to be 0.993, and substantiated the strength of this correlation.

- *Sensitivity of real-time PCR and HRM analysis*

After conducting this experiment on pure bacteria, regarding the primer pair used here and the temperature schedule observed, real-time PCR and HRM analysis could detect 200 bacterial cells using the designed primer common for *S. enteritidis* and *S. typhimurium*.

In the present study, the REAL TIME-PCR assay was conducted together with the melting curve analysis to detect *S. enteritidis*, *S. typhimurium*, or their mixture in raw egg samples that were contaminated manually. Our assay includes a pre-enrichment stage of culturing in buffer peptone water (BPW) and a subsequent enrichment stage in the form of Rappaport culture. After counting of the injected bacteria, DNA extraction was carried out, and to continue the steps of the assay, the samples were placed in the REAL TIME-PCR block of the cycler. The total duration of the assay was approximately 24 hours, which is much faster as compared to the traditional culture methods, which may take up to 5 days (Hein *et al*., 2006).

In general, it can be deduced that the sensitivity and effectiveness of REAL TIME-PCR have increased through pre-enrichment and enrichment stages for *Salmonella* cells. Accordingly, Wolffs *et al*. (2006) used REAL TIME-PCR and SYBR Green to detect *Salmonella* and obtained a concentration of 7.5×10^2 CFU/ml. The correlation for enriched samples was .998, and .989 for whole cells (Wolffs *et al*., 2006). REAL TIME-PCR in conjunction with HRM analysis involves a simpler procedure as compared with probe-based PCR and hence does not require the design of more complex probes. In the probe-based REAL TIME-PCR analysis, nonspecific binding of a probe can be considered a positive result, while in HRM analysis, the nonspecific binding is easily distinguished and analyzed.

The results of the present study are in line with many other studies (De Medici *et al*., 2003; Bratchikov and Mauricas, 2011). The sensitivity achieved in this study (based on REAL TIME-PCR testing for *S. enteritidis* and *S. typhimurium* and their mixture) is at least 200 bacterial cells detectable by the quantitative PCR assay with the designed primer; this sensitivity level is better (lower) than that in other studies (Singh and Mustapha, 2014; Seo and Brackett, 2005; Zhang *et al.*, 2011) Comparing the PCR and REAL TIME-PCR methods, Chen *et al.* (2000) reported that the sensitivity of the latter is 2 CFU/ml (Chen *et al*., 2000).

In another study, which was conducted by Medici *et al.* (2003) on *S. enteritidis* using REAL TIME-PCR with SYBR Green and HRM analysis, the limit of detection was found to be less than 10^3 CFU/ml, while the data correlation was .9767 (R^2 = .9767). T_m

analysis yielded 82.56°C ± .22°C for *S. enteritidis*, whereas in the present study, this figure was 90.42°C ± 1.06°C for pure *Salmonella* (De Medici *et al*., 2003).

Vanblerk *et al*. (2011) utilized amplicon size of 284 bp for detection of *Salmonella* in water samples, and T_m was found to be 83.64°C ± .28°C. The number of cycles from denaturation to elongation was adjusted to 45, and the annealing temperature decrease for HRM analysis was performed down to 60°C (van Blerk *et al*., 2011). In comparison, in the present work, the amplicon size was 130 bp, and in HRM analysis, the significance range contained 90% of results, region 1 normalization showed temperatures between 86.54°C and 87.18°C, and region 2 normalization yielded temperatures between 90.13°C and 90.71°C. It is worth noting that after the elongation stage, the temperature was decreased down to 50°C, and the number of cycles in the range mentioned above was 25. In fact, based on the temperature information outlined in Tables 2 and 3, initially, the HRM analysis was performed based on the information from Table 2, and consequently, the assay could detect the mixed bacteria and construct a *Salmonella* graph. By contrast, in the second part of the experiment with a new temperature profile, these three types of samples were well detectable and distinguishable after normalization of the graphs.

Singh (2014), among many other scientists, used REAL TIME-PCR in the multiplex format to detect 41 species of *Salmonella*, among which 8 were artificially injected into biological samples. The resulting sensitivity was 290 CFU/ml. The limit of detection of this method for the samples that were not enriched was 10^4 CFU/ml, and for the samples that were enriched for 6 hours, it was 10 CFU/ml (Singh and Mustapha, 2014). As another example of studies in this field, we can refer to the study by Seo (2005) for the detection and calculation of *Salmonella* cell concentrations using quantitative PCR, where the limit of detection was 10^3 (Seo and Brackett, 2005).

Among the factors affecting T_m, there are such parameters as size, CG content (Druml and Cichna-Markl, 2014; Bratchikov and Mauricas, 2011), complexity of the amplified sequence, as well as the melting ratio. On the other hand, theoretically speaking, T_m is dependent on CG content (Bratchikov and Mauricas, 2011). The cycler's optics and the interpretation of the fluorescent signals by the cycler-associated software also have a strong influence on apparent T_m (Xiao *et al*., 2014). The temperature and concentration of the magnesium ion play a major role in stabilization and annealing of the probe. In case the cycler does not function properly and causes an unwanted temperature difference of ~3°C, vague results can be obtained at the elongation and annealing stages. In most cases, scientists have used relatively similar temperature–time profiles and in all of them, the temperatures used were in a narrow range. Reed (2007) and Vossen (2009) believe that the quantity of DNA and its purity are other significant factors for the detection and identification of a target sequence (Reed *et al*., 2007; Vossen *et al*., 2009). As mentioned earlier, because the amplicon length affects the HRM analysis sensitivity, the length of amplicons should not exceed 300 bp (Druml and Cichna-Markl, 2014), and in line with this notion, this length was 130 bp in the present study.

Conclusion

In the present study, it was concluded that the egg samples randomly obtained in the market were devoid of any contamination with *S. enteritidis* or *S. typhimurium*. Therefore, this study suggests that the REAL TIME-PCR and HRM analysis can detect

foodborne pathogens, namely *S. typhimurium* in eggs, in less than 24 hours. In fact, HRM analysis is a reproducible ideal subtyping method that does not take much time and is easily interpretable and usable in a laboratory. It should also be noted that the optimization of the reaction using HRM analysis, optimization of reaction performance and conditions, the use of suitable primers, and increasing the enrichment time can sharply reduce the limit of detection (i.e., the number of bacteria detected). Meanwhile, the shortest time required for identification of *Salmonella* species is between 47 and 72 hours.

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