Application of Real-time PCR for Rapid Petection of *Salmonella* spp., *Salmonella enterica* ser. Typhimurium and Enteritidis in Food Samples of Animal Origin without Pre-enrichment and with Pre-enrichment

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Abstract
The aim of this study was follow the contamination of milk and meat products with *Salmonella* spp. by using the Step One real-time PCR. We used the PrepSEQ Rapid Spin Sample Preparation Kit for isolation of DNA and Power SYBR Green PCR Master Mix for pursuance the real-time PCR (Applied Biosystems). We were observing the presence of genes stn (*Salmonella enterica* ser. Typhimurium DT096), sef and pef (*Salmonella enterica* ser. Enteritidis SE7). We could detect strain of *Salmonella* spp. in the investigated samples without pre-enrichment and after pre-enrichment. Our results indicated that the Step One real-time PCR assay developed in this study could sensitively detect *Salmonella* spp. in food samples of animal origin (swabs). This Step One real-time PCR assay is extremely useful for any laboratory in possession of a real-time PCR. It is a fast, reproducible, simple, specific and sensitive way to detect nucleic acids, which could be used in clinical diagnostic tests in the future. Our results indicated that the Step One real-time PCR assay developed in this study could sensitively detect *Salmonella* spp. in ready to eat food. Thus, these results proved real-time PCR to be useful as a rapid diagnostic test for the direct detection of pathogens in food of animal origin, without the need of pre-enrichment steps.

Keywords: pre-enrichment, real-time PCR, Salmonella spp., SYBR Green

1. Introduction

Food poisoning is usually caused by the consumption of contaminated food or water containing various bacteria, viruses, parasites or toxins of biochemical or chemical nature. As salmonellae are ubiquitous in the environment, they are common causative agents of food poisoning. Foodborne illnesses caused by Salmonella are common in the United States and in many European countries, and have always been a significant health burden worldwide [1-3].
In 2010, about 5300 ready-to-eat food samples were scheduled for *Salmonella* detection by PHLSB in the Food Surveillance Programme. Traditionally, labor-intensive and time-consuming conventional standard culture methods, which usually require at least 3 working days to generate

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the results, have always been employed by many laboratories to detect *Salmonella* in foods [4].

PCR methods for *Salmonella* detection employ primer pairs specific or complementary only to DNA sequences of *Salmonella* spp. genes. During PCR amplification, the *Salmonella* DNA sequence flanked by a pair of the species-specific primers is amplified with DNA polymerase. After 30 to 40 cycles of PCR amplification, the resulting PCR products, which are of the same size, could be easily detected by agarose gel electrophoresis. DNA sequences of other species, if present in the sample, would not be amplified as the primers could not anneal to the sequences of non-*Salmonella* DNA under the selected PCR conditions. Some early conventional gel-based PCR methods had been developed based on the sequence of different *Salmonella* genes, such as, the major fimbrial subunit encoding gene (fimA), a *Salmonella* invasion gene (invA), and a virulence gene (spvC) [5-6].

A recent comparison was done on the effect of three different enrichment media, namely, buffered peptone water, tryptic soy broth, and universal preenrichment broth, on the real-time SYBR Green PCR detection of *S. enterica* from fresh produce [7]. An earlier study reported that the performance of a TaqMan-based quantitative RTq PCR targeting the invA mRNA of Salmonella was comparable to that of the conventional cultural method when a pre-enrichment step was employed to enhance the RT-qPCR detection limit [8].

Growth of non-target background microflora in sample enrichments that inhibits PCR assays necessitates the development of other sample preparation methods such as those that can directly separate the target bacteria from the background microflora. A two-step filtration combined with real-time PCR assay was developed for direct quantification of salmonellae in chicken rinse samples [9].

However, presently available commercialized nucleic acid-based assays, including the more sensitive fluorogenic real-time PCR methods, for *Salmonella* detection in foods seldom have a sensitivity of 1 bacterial cell per 25 g of food. To enhance the detection limit for *Salmonella* detection, it would be useful to include a pre-enrichment step to allowmultiplication of target bacterial cells during sample preparation. A pre-enrichment step can also help reducing the risk of amplifying sequences from dead target cells in the sample. The molecular-beacon real-time PCR was able to detect as few as 4 CFU of *Salmonella* cells per 25 g artificially contaminated fruits and vegetables after 16 h of pre-enrichment in buffered peptone broth [10].

2. Materials and methods

Food Samples

Total of 8 food samples were used in this study. Samples were obtained by taking of swabs from the inside of the food of animal origin. As the aim of investigation were observed milk products and meat products. After sampling, procedure shown in the Scheme 1 was used.

Bacterial Strains and DNA Extraction

As a pre-preparation step for the Step One real-time PCR, DNA extraction was performed using DNA extraction method: PrepSEQ Rapid Spin Sample Preparation Kit (Applied Biosystems, USA).

General Sample Preparation Protocol

Sample of 750 µL was loaded onto the spin column and microcentrifuged for 3 minutes at maximum speed. Supernatant was discarded and 50 µL of Lysis Buffer was added to the pellet. Samples were incubated for 10 minutes at 95°C.

Primers and Real-time PCR

The molecular diagnosis of *Salmonella* spp., were focused on the presence of primers: *stn* (*Salmonella enterica* ser. Typhimurium DT096), *sef* and *pef* (*Salmonella enterica* ser. Enteritidis SE7) genes.

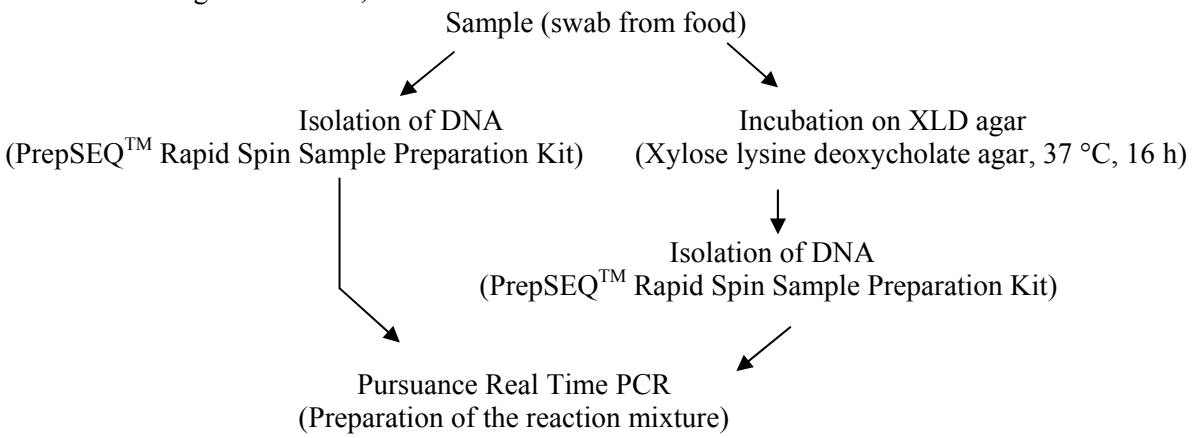
Reaction mixture: 15 µl SYBR Green PCR, Master Mix,
1.5µl of primers (F + R),
3µl samples after isolation,
10.5µl of deionized water.

The PCR primers and reaction conditions were as follows: *stn* gene:

Primers*stn*P15 'TGT TTGCGCCACTATTGGCAACC3'and*stn*M13 5'ATT GTTAACCTCTCGTCCCCG3'.The

reaction consists of 25cycles of denaturation (94°C, 1 min), annealing (59°C, 1 min), elongation (72°C, 1 min).

Sef gene: primer *sef* of C5 'GCG AAAACCAATGTAACTGCG3'and the head of C5 'CCC ACCAGAAACATTCCCCAT3'. The reaction consists of 25cycles of denaturation (94°C, 1 min), annealing (55°C, 1 min), elongation (72°C, 1 min).
Pef gene: primer pefA15 'TGT GCTTTCCGCTGTGCT3' and A25 'CAG CGGTGCATTTGATTCC3'. The reaction consists of 25 cycles of denaturation (94°C, 55 s), annealing (55°C, 55 s), elongation (72°C, 55 s).
Data were collected during each elongation step. PCR products were detected by monitoring of the increase in fluorescence of the reporter dye at each PCR cycle. TaqMan® probes labeled with both a fluorophore and a quencher dye were used in real-time PCR assays to detect amplification of specific DNA targets. FAM™, which has an emission of 520 nm, has become the most commonly used fluorophore for single plex qPCR reactions. TAMRA™ will efficiently quench the fluorescence of FAM™, until the probe hybridizes to the target and is cleaved by the 5'exonuclease activity of the polymerase. We used three fluorophore detection chemistries that include FAM™ and VIC® dye-labeled TaqMan® MGB probe-based assays, VIC® and TAMRA™ dye-labeled probe-based assays and ROX™ as passive reference dye. PCR products were detected by monitoring of the increase in fluorescence of the reporter dye at each PCR cycle.
Applied Biosystems software plots the normalized reporter signal, ΔRn, (reporter signal minus background) against the number of amplification cycles and also determines the threshold cycle (Ct) value i.e. the PCR cycle number at which fluorescence increases above a defined threshold level were used.



Scheme 1. Progress after taking of the samples

3. Results and discussion

The most sensitive detection of *Salmonella* spp. was obtained using PrepSEQ™ Rapid Spin Sample Preparation Kit and SYBR Green PCR Master Mix compatible with StepOne™ Systems, was less time-consuming than the other methods and relatively easy to use.
Thus, the PCR-based detection of bacteria depends on the efficiency of DNA extraction procedure used to prepare the template DNA. In the investigated samples before incubation we could detect strain of *Salmonella* spp. in all samples, as well as positive control (PC) as shown in Table 1 and Figure 1. The (Ct) value of positive *Salmonella* samples were from 15.03 to 17.97 and PC (Ct) value was 32.23. In the samples after 16 hours incubation we could detect strain of *Salmonella* spp. in all samples, as well as in PCas shown in Table 1. The (Ct) value of positive *Salmonella* samples was from 27.63 to 32.23 and PC (Ct) value was 17.97.
Pre-enriched meats had CT values of 35.32 or more, while post-enriched meats had CT values of 14.41 to 22.23. When 1.45±0.21 log10 CFU/mL of *S.* Typhimurium and 1.65±0.07 log10 CFU/mL of *S.* Enteritidis were spiked into meats,
the multiplex real-time PCR assay detected bacteria in all conditions (beef, pork, pre-enriched, and post-enriched) [11].

Table 1. Results of real-time PCR (Ct)

Sample + Primer	Without pre-enrichment	After pre-enrichment
Tourist salami + sef	30.99	16.77
Sour Cream + sef	31.41	17.10
Ipel' sausage + sef	31.61	15.91
Tourist salami + pef	31.21	15.03
Sour Cream + pef	31.24	16.20
Ipel' sausage + stn	31.42	17.97
Sour Cream + stn	32.23	16.69
Positive Control	27.63	16,96
Negative Control	negative	negative

Ct - threshold cycle value

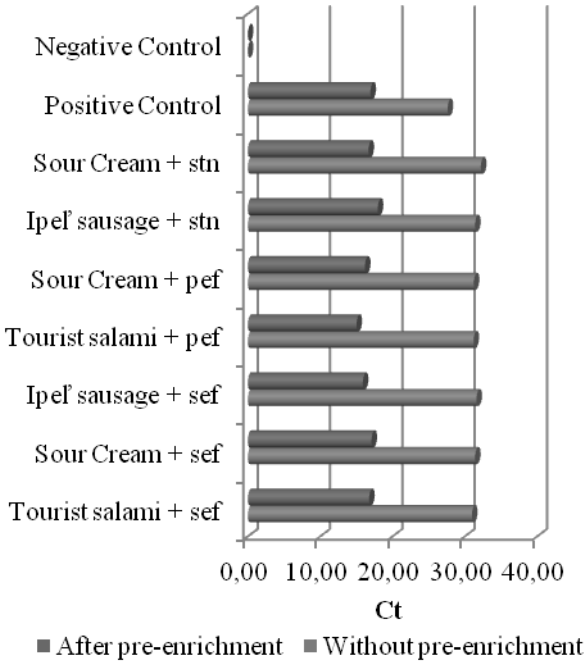


Figure 1. Real-time PCR detection of *Salmonella* spp.

The multiplex real-time PCR developed in this study was the first to detect all *Salmonella* spp. possibly related with meats and to differentiate simultaneously *S.* Typhimurium from *S.* Enteritidis in meats. Previously, real-time PCR assays had been applied for *Salmonella* spp. and other food-borne pathogens [12-14]. Although there has been a fall in the number of human Salmonella infections in Europe over the past number of years [15], foodborne infection from Salmonella continues to pose a great risk to public health. It is widely believed that pathogen reduction in animals, efficient Quality Control Systems (e.g. HACCP principles and GHP) and Quality Assurance (QA) at all stages of the food chain from ''farm to fork" is the most effective way to prevent the spread of infection via food [15].

Possible biological contaminants such as blood and fats present on animal carcasses which can cause PCR inhibition are also diluted. The bacterial cell pellet was washed in PBS prior to DNA isolation to reduce the concentration of enrichment medium which may also cause PCR inhibition [16].

Whereas most of the target genes used for PCR detection of foodborne pathogens encode proteins involved in virulence and were identified as the result of many years of work involving gene/protein structure–function studies, the progress in computational genomics has led the way to more efficient and customized mining of genomes for species-specific nucleotide sequences. Several software packages for such data mining have been developed based upon sequence alignment [17-18].

4. Conclusions

Testing laboratories that analyze samples routinely for food-borne pathogens, e.g., S*almonella*, usually prefer the application of cost

effective test methods with high productivity as well as analytical flexibility. Advance in technologies has enabled the development of a variety of rapid test methods for testing food-borne pathogens.

Based on the conventional PCR methods, automated fluorogenic and quantitative real-time PCR kits and platforms have been invented and become available on the market. The rapid real-time PCR based method was performed very well compared to the conventional method. It is fast,

reproducible, simple, specific and sensitive way to detect nucleic acids, which could be used in clinical diagnostic tests in the future. Conventional PCR methods need amplification product separation by gel electrophoresis following by hybridization with a probe. These time-consuming protocols are replaced nowadays by more convenient and rapid real-time PCR assays.

Our results indicate that the Step One real-time PCR assay developed in this study could sensitively detect *Salmonella* spp. in ready to eat food. This could prevent many people from becoming infected with *Salmonella*, it could benefit for food manufacturing companies by extending their product's shelf-life by several days and saving them the cost of warehousing their food products while awaiting pathogen testing results.

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References

1. Magnino, S., Colin, P., Dei-Cas, E., Madsen, M., McLauchlin, J., Nöckler, K., et al., Biological risks associated with consumption of reptile products. International Journal of Food Microbiology, 2009, 134, 163–175

2. Davies, R.H., Wales, A.D., Investigations into Salmonella contamination in poultry feedmills in the United Kingdom, Journal of Applied Microbiology, 2010, 109, 1430–1440

3. Patel, M.K., Chen, S., Pringle, J., Russo, E., Viñaras, J., Weiss, J., et al., A prolonged outbreak of Salmonella Montevideo infections associated with multiple locations of a restaurant chain in Phoenix, Arizona, 2008. Journal of Food Protection, 2010, 73, 1858–1863

4. Cheung, P.Y., Kwok, K.K., Kam, K.M., Application of BAX system, Tecra Unique™ Salmonella test, and a conventional culture method for the detection of *Salmonella* in ready-to-eat and raw foods, Journal of Applied Microbiology, 2007, 103, 219–227

5. Cohen, H.J., Mechanda, S.M., Lin, W., PCR amplification of the fim A gene sequence of *Salmonella typhimurium*, a specific method for detection of *Salmonella* spp., Applied and Environmental Microbiology, 1996, 62, 4303–4308

6. Swamy, S.C., Barnhart, H.M., Lee, M.D., Dreesen, D.W., Virulence of determinants inv A and spv C in salmonellae isolated from poultry products, wastewater, and human sources., Applied and Environmental Microbiology, 1996, 62, 3768–3771

7. Gorski, L., Liang, A.S., Effect of enrichment medium on real-time detection of *Salmonella enterica* from lettuce and tomato enrichment cultures, Journal of Food Protection, 2010, 73, 1047–1056

8. González-Escalona, N., Hammack, T.S., Russell, M., Jacobson, A.P., De Jesus, A.J., Brown, E.W., et al., Detection of live *Salmonella* sp. cells in produce by a TaqMan based quantitative reverse transcriptase real-time PCR targeting inv A mRNA, Applied and Environmental Microbiology, 2009, 75, 3714–3720

9. Wolffs, P.F.G., Glencross, K., Thibaudeau, R., Griffiths, M.W. Direct quantitation and detection of Salmonellae in biological samples without enrichment, using two step filtration and real-time PCR, Applied and Environmental Microbiology, 2006, 72, 3896–3900

10. Liming, S.H., Bhagwat, A.A., Application of a molecular beacon-real-time PCR technology to detect *Salmonella* species contaminating fruits and vegetables, International Journal of Food Microbiology, 2006, 95, 177–187

11. Higgins, J.A., Jenkins, M.C., Shelton, D.R., Fayer, R., Karns, J.S., Rapid extraction of DNA from *Escherichia coli* and *Cryptosporidium parvum* for use in PCR, Applied Environmental Microbiology 2001, 67, 5321–5324

12. Malorny, B., Paccassoni, E., Fach, P., Bunge, C., Martin, A., Helmuth, R. Diagnostic real-time PCR for detection of *Salmonella* in food, Applied Environmental Microbiology, 2004, 70, 7046-7052

13. Rossmanith, P., Krassnig, M., Wagner, M., Hein, I. Detection of *Listeria monocytogenes* in food using a combined enrichment/real-time PCR method targeting the *prfA*gene, Research Microbiology, 2006, 157, 763-771

14. Sails, A D., Fox, A.J., Bolton, F.J., Wareing, D.R., Greenway, D.L., A real-time PCR assay for the detection of *Campylobacter jejuni* in foods after enrichment culture, Applied Environmental Microbiology, 2003, 69, 1383-1390

15. Anonymous. Regulation (EC) 2160/2003 of the European Parliament and of the Council of 17 November 2003 on the control of *Salmonella* and other specified food-borne zoonotic agents

16. Rodriguez, Lazaro, D., Hernandez, M., Molecular methodology in food microbiology diagnostics: Trends and current challenges. IUFoST: IUFoST World Congress., 2006, pp.1085–1099

17. Lu, G., Jiang, L., Helikar, R.M., Rowley, T.W., Zhang, L., Chen, X., Moriyama, E.N., Genome Blast: a web tool for small genome comparison, BMC Bioinformatics, 2006, 8, 18

18. Yao, J., Lin, H., Doddapaneni, H., Civerolo, E.L. Way Comp: a genome-wide sequence comparison tool for multiple strains/species of phylogenetically related microorganisms, Silico Biology, 2007, 7, 195–200