Short Paper

Determination of RT-PCR detection limit of live and dead *Salmonella* cells in raw and sterilized milk

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Summary

The objective of the current study was to evaluate the reproducibility of a reverse transcriptase PCR (RT-PCR)-based technique to differentiate viable and dead *Salmonella* cells in raw and sterilized milk. The microorganism was initially inoculated into the milk samples followed by incubating at 37°C for 4 h prior to inactivation by heat at 80°C for 10 min. The treated and non-treated samples were subsequently monitored using both PCR and RT-PCR, *in vitro*. Following 4 h incubation, the *invA* gene of *Salmonella* was clearly amplified by RT-PCR, while no band was detected in the heated samples. On the other hand, using the conventional PCR, it was possible to amplify the gene in both samples. Our results may suggest an important application of the RT-PCR technique, especially when the number of live organisms is an imperative factor to produce food borne infections and to establish a detection limit of the test.

Key words: *Salmonella*, Reverse transcriptase PCR, Milk

Introduction

Detection of non-viable bacterial cells has been previously addressed by conventional based techniques PCR (Simpkins *et al*., 2000). One of the major disadvantages of such techniques is that they cannot distinguish between live and dead cells (Gonzalez-Escalona *et al*., 2009).

An RNA-based RT-PCR method for the detection of *Salmonella enterica* was developed (Simpkins *et al*., 2000; Miller *et al*., 2011). The usefulness of mRNA in detecting cell viability has been proposed (Bej *et al*., 1991) and thus RT-PCR, a reverse transcription copy of DNA, has been used (Vaitilinghom *et al*., 1998). Due to the high sensitivity of PCR, it may detect traces of genomic DNA to produce false positive results in a viability assay (Simpkins *et al*., 2000). As such, a special real-time RT-PCR and TaqMan quantitative real-time PCR assays were developed as rapid and sensitive techniques to detect *Salmonella typhimurium* within 24 h, the assay shows promise for routine testing and monitoring *Salmonella* (Gonzalez-Escalona *et al*., 2009; Techathuvanan *et al*., 2010). The highly conserved region of *invA* gene presents in a wide range of *Salmonella* serotypes including all subspecies in many food stuff used as was formerly described (Jyoti *et al*., 2010).

Distinguishing between live and dead microorganisms is of a great concern in food microbiology, where initial knowledge of the presence of live microorganisms in foods in which viable microbial cells can be dramatically increased during different incubation times is necessary. This condition is appropriate to show the low number of live *Salmonella*, which subsequently may induce the food contamination. Low numbers (15-100 CFU) of *Salmonella* in food or water may pose a public health risk. The aim of the present work was to introduce a quick and reliable technique to detect the live *Salmonella* in raw and sterilized milk.

Materials and Methods

Bacterial preparation

According to the correlation between viable cell count of *Salmonella typhimurium* and optical density, the bacterial suspension was added to the tryptic soy broth (TSB), skimmed UHT milk and skimmed raw milk before performing a tenfold serial dilution with the same media. The final concentration was 10^8 CFU/ml to 10^{-1} CFU/ml. Each dilution was divided into four portions and treated as follows:

1- Used for DNA and RNA extraction and bacterial count.

2- Enriched at 35°C for 4 h and then used for DNA and RNA extraction and bacterial count.

3- Heated in water bath at 80°C for 10 min, used for DNA and RNA extraction and examined for viability.

4- Heated in water bath at 80°C for 10 min, enriched at 35°C for 4 h and used for DNA and RNA extraction and examined for viability

To determine the bacterial count, 1 ml of each sample was poured into McConkey agar media in duplicate and then incubated at 35°C for 24 h (if necessary, tenfold serial dilution was performed using 0.1% peptone water).

The viability was examined by 1 ml of each sample inoculated into 10 ml of TSB and incubated at 35°C for 48 h followed by being subcultured onto the McConkey agar.

All experiments were performed in triplicate, independently.

DNA extraction

DNA was extracted from 150 µl of each dilution using a Qiagen extraction kit, as recommended by the manufacturer.

RNA extraction

The pellet of 200 µl centrifuged samples was homogenized in Eppendorf tubes in 300 µl of RNX-plus solution (CinnaGen, Iran) by vortexing and further 700 µl of RNX-plus solution was added. The tubes were placed at room temperature and after 3-5 min, 200 µl of chloroform was added before centrifuging at 12,000 g for 5 min at 4°C. From each tube, 500 µl of the clear supernatant was collected and transferred into a new tube. To each tube, 500 µl isopropanol was then added and kept at 4°C for 15 min. The tubes were then centrifuged at 12,000 g for 10 min at 4°C. The supernatants were carefully discarded, the bottom pellet washed using 80% cold ethanol, and centrifuged at 12,000 g for 7 min at 4°C. After discarding the supernatants, the clean RNA was finally re-suspended in 50 µl elution buffer (Qiagen, Germany, Cat. No. 28704) and kept in -70°C for further use.

RT-PCR

1. Complementary DNA (cDNA) synthesis

Reverse primer was used for synthesis of the first strand of cDNA. All chemicals and enzymes were provided by Fermentas (Lithuania). Eight µl RNA, 1 µl reverse primer and 2 µl distilled water were mixed and heated at 65-70°C for 10 min. A mixture consisting of 4 µl of 5 X reaction buffer, 2 µl dNTPs mix (10 mM) and 1 µl Riblock RNase inhibitor (40 units) was added, and the reactions were incubated at 37°C for 5 min. One µl (200 units) of Revert Aid^{TM} M-MuLV reverse transcriptase enzyme was then added to each reaction. The tubes were placed in water bath at 37°C for 15-20 min. The temperature was raised by 1°C every 15-20 min until it reached to 42°C, in which the reaction was incubated for 30 min. The prepared cDNA was kept at -20°C for further use. cDNA was quantified spectrophotometrically for further use.

2. PCR

Primers: Based on the *invA* gene (accession No. CP003416.1) of *Salmonella* strains, specific primers, S139 and S141 with the following sequence were used, 5´- GTG AAA TTA TCG CCA CGT TCG GGC AA-3´ and 5´- TCA TCG CAC CGT CAA AGG AAC C-3´. The predicted size of amplicons was 285 bp for the genus *Salmonella*. PCR was carried out as previously described (Zahraei-Salehi *et al*., 2005).

Results

invA gene and *invA* mRNA were monitored in all the treatments. However, when different dilutions of the microorganisms were applied in TSB, raw and sterilized milk, the DNA was detected in all of the preparations, including live or dead cells, either before or after the enrichment using conventional PCR.

Table 1 implied that when the aliquot of live *Salmonella* in TSB was used, 5×10^0 and 5×10^2 CFU/ml were detected using RT-PCR, in enriched and non-treated samples, respectively. In the case of heated cells at 80°C for 10 min, 5×10^0 CFU/ml DNA was detected by PCR, whilst no RNA was confirmed using RT-PCR. Furthermore, when the inoculated UHT and

Table 1: Detection limit of live and dead *Salmonella* cells *(invA* gene) in TSB and milk inoculated with *Salmonella typhimurium* (ATCC, 35986)

Media	Treatment		Detection limit	
	Heating (80°C for 10 min)	Post heating enrichment (35°C for 4 h)	DNA (CFU/ml)	RNA (CFU/ml)
TSB	NP	NP	5×10^0	5×10^2
	NP	P	5×10^0	5×10^0
	P	NP	5×10^0	ND
	P	P	5×10^0	ND
SUHTM	NP	NP	1×10^4	1×10^5
	NP	P	1×10^4	1×10^4
	P	NP	1×10^4	ND
	P	P	1×10^4	ND
SRM	NP	NP	1×10^3	1×10^4
	NP	P	1×10^3	1×10^3
	P	NP	1×10^3	ND
	P	P	1×10^3	ND

TSB: Tryptic soy broth, SUHTM: Skimmed ultra high temperature sterilized milk, SRM: Skimmed raw milk, NP: Not performed, P: Performed, and ND: Not detected. n=3, results of three replicates were similar

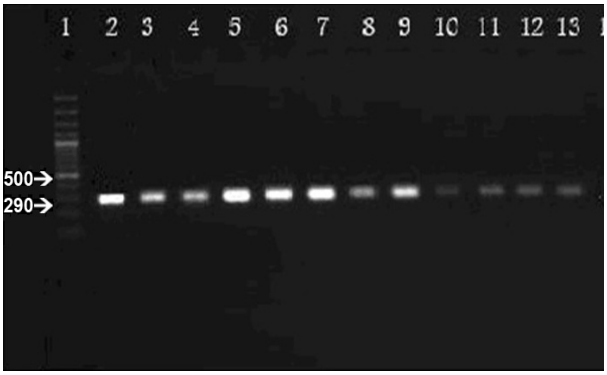

Fig. 1: Detection of *invA* gene from *Salmonella*: comparison between live and dead cells from TSB using conventional PCR (Lanes 1 and 18: 100 bp marker, even numbered lanes from Lanes 2 to 16 represent dead bacterial cells, odd numbered Lanes from 3 to 17 represent live bacterial cells. Lanes 2 and 3: 5×10^5, Lanes 4 and 5: 5×10^4, Lanes 6 and 7: 5×10^3, Lanes 8 and 9: 5×10^2, Lanes 10 and 11: 5×10^1, Lanes 12 and 13: 5×10^0, Lanes 14 and 15: 5×10^{-1}, and Lanes 16 and 17: 5×10^{-2} CFU/ml

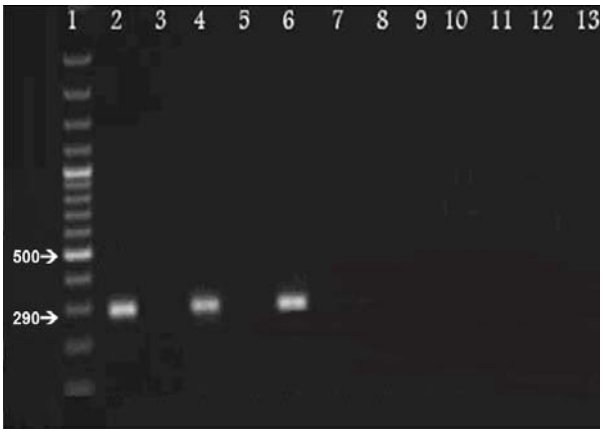

Fig. 2: Detection of live *Salmonella* (*invA* gene): results of RT-PCR reactions on the enriched raw milk at 35°C for 4 h. Lanes 1 and 18: 100 bp marker, even numbered lanes from Lanes 2 to 16 represent live bacterial cells, odd numbered Lanes from 3 to 17 represent dead bacterial cells. Lanes 2 and 3: 1×10^5, Lanes 4 and 5: 1×10^4, Lanes 6 and 7: 1×10^3, Lanes 8 and 9: 1×10^2, Lanes 10 and 11: 1×10^1, Lanes 12 and 13: 1×10^0, Lanes 14 and 15: 1 and 10^{-1}, Lanes 16 and 17: 1×10^{-2} CFU/ml

raw milk were enriched at 35°C for 4 h followed by heating at 80°C for 10 min., 1×10^4 CFU/ml and 1×10^3 CFU/ml were detected by the PCR, respectively. In contrast, no RNA was detected using RT-PCR (Figs. 1 and 2). In each experiment, results of the three replicates were similar.

Discussion

Various numbers of molecular approaches such as RT-PCR have shown the advantages in the differentiation between live and dead microorganisms by *in vitro* models (Sazbo and MacKey, 1999; Simpkins *et al*., 2000; Rijpens *et al*., 2002; Postollec *et al*., 2011).

In the enriched bacterial suspension after heating at 80°C for 10 min, 5×10^0 CFU/ml DNA was detected by PCR, whilst no RNA was confirmed using RT-PCR. After heating at 80°C for 10 min, in the sterilized and/or raw milk, the amount of bacteria confirmed by conventional PCR were 1×10^4 and 1×10^3, respectively, while no RNA was detected. This might be related to the lower sensitivity of the assay to detect DNA in the presence of some inhibitory agents compared to TSB.

Even though the quantitative analysis of food-borne pathogens is a major issue that is extensively developed and has become available in several real-time formats (Foy and Parkes, 2001). The RT-PCR technique has been applied to detect the enterotoxin producer *Salmonella* cells (Dinjus *et al*., 1997). However, the implementation of such quantitative techniques is hampered by their lack of ability to distinguish between viable and dead cells. The most intense RT-PCR amplicon was from exponential phase cells (9 h at 37°C) was previously demonstrated (Sazbo and MacKey, 1999). Moreover, it was also confirmed that the sensitivity of the RT-PCR to detect *saf A* was 10,000-fold greater than that of late stationary phase. Here, we observed an intense band following 4 h incubation at 35°C. Different studies have previously been performed to detect the viable food borne pathogens using RT-PCR (Vaitilinghom *et al*., 1998; Sazbo and MacKey, 1999; Rijpens *et al*., 2002). However, none of them have employed the technique based on confirmation of the number of *Salmonella* in milk.

In the present study, an *in vitro* model was designed using RT-PCR assay in order to differentiate live and dead *Salmonella* in both raw and sterilized milk and to find out the detection limit of the test.

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