Evaluation of a Real-Time PCR Multiplex Assay For Detecting Foodborne Pathogenic Bacteria

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Asbtract

Molecular biological methods are feasible, quick and reliable tools of detecting major foodborne pathogens in food quality and safety monitoring. Among them, *real-time* PCR application is one of the testing platforms that optimally meets the criteria of performance against high throughput screening of microorganisms. The objective of this study was to evaluate the performance of a *real-time* PCR fourplex assay in simultaneous detection of major foodborne pathogenic bacteria, including *Escherichia coli*, *Staphylococcus aureus*, *Listeria monocytogenes* and *Salmonella* spp. To do this, the reference variants were inoculated to UHT milk sample individually as well as a cocktail containing them at all hands. All the spiked suspensions were initially exposed to pre-enrichment in buffered peptone water. Subsequently, 10-fold serial dilutions were prepared from them. The serially diluted suspensions were then transferred to Plate Count (PC) agar plates, followed by an aerobic incubation under the conditions according to the mannufacturer's instructions. After that, the plates expressing a viable count of 1-10 CFU/ml, 10-100 CFU/ml and 100-1000 CFU/ml were selected. The DNAs extracted by Eurofins GENESpin DNA isolation kit were subjected to a *real-time* PCR fourplex application using PowerChek™ Pathogen 4-plex detection kit (Kogene Biotech, South Korea). The screening results revealed that the tested multiplex assay simultaneously exhibited its best performance as triplex for Sta, Lm and Sal in a concentration of 10-100 CFU/ml, excluding Ec. To conclude, detection and differentiation of multiplex bacteria are not yet optimized due to some technical limitations, and still evolving.

Keywords: *Food, Multiplex assay, real-time PCR, Sensitivity.*

1. Introduction

Foodborne diseases are major public health problem worldwide over the last 20 years. Nowadays, almost 25% of the people in the World is considered to be at a higher risk for foodborne diseases [1].

A wide variety of bacteria may be present in the foods with the microbial status being influenced by animal health, environment and production methods. Among them, *Escherichia coli* O157:H7, *Staphylococcus aureus*, *Listeria monocytogenes*, and *Salmonella* spp. are responsible for most of the foodborne disease outbreaks [2]. Though standards to identify and monitor the foodborne high-risk carrying bacteria were set by national and international authorities to ensure food quality and safety [3].

Conventional bacterial testing methods are based on using a specific media for

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enumeration and isolation of viable bacterial cells occurring from the foods. These methods are sensitive, inexpensive, and yield both qualitative and quantitative information about the microorganisms [4].

Currently, the food safety practices require high throughput screening of an diverse array of foods. In order for a testing format to be feasible tool in the food quality and safety monitoring, it must have reproducible sensitivity and marked specificity as well as being fast, low-cost per assay, acceptible, and ease of use by the staff. However, the dulture-based methods can not optimally meet these performance criteria [5].

Numerous conventional methods were developed to detect or confirm the foodborne pathogen bacteria. However, it is not still clear which tests are the most sensitive [6]. Because, conventional methods can not detect one-third of these high-risk carrying bacteria in the foods [7]. In this case, genotypic testing is further needed [8].

Rapid identification of pathogens may prevent foodborne diseases through better control of foods. Pathogenic bacteria that were previously isolated and identified by conventional testing procedures can be easily detected quickly and reliably by rapid testing methodologies, including molecular biological assays. However, DNA-based techniques can be adversely affected by interfering substances in the sample or lack the sensitivity needed to detect bacteria in very low levels [9]. By contrast, sensitivity is very important because a single foodborne pathogen has the risk to cause infection [1].

Real-time PCR is a polymerase chain reaction process in which a target DNA is amplified and quantified simultaneously within a reaction. This method uses specific primer set, one or two probes and/or fluorescent dye to get detection signals for the increase of detection specificity and design of multiplex detection methods [10]. In *real-time* PCR, the amplified DNA is detected in real time as the reaction progresses instead of at the reaction end [11].

A *real-time* PCR multiplex assay ideally performs the simultaneous amplification and detection of more than one target sequence in a single reaction without influencing the cross-talk and loss of sensitivity [12]. In some *real-time* PCR assays, four different amplification products (fourplex) can be distinguished in a single tube. This situation significantly leads to the reduction of hands-on time [13]. Because, *in vitro* amplification-based detection of genetic elements is much more rapid and sensitive than conventional method [14].

The objective of this study was to evaluate the performance of a real-time PCR fourplex assay in simultaneous detection of major foodborne pathogenic bacteria, including *Escherichia coli*, *Staphylococcus aureus*, *Listeria monocytogenes* and *Salmonella* spp.

2. Materıal And Methods

Reference cultures

As the standardized cultures, *Escherichia* (*E.*) *coli* O157:H7 ATCC 25922, *Staphylococcus* (*S.*) *aureus* ATCC13565, *Listeria* (*L.*) *monocytogenes* ATCC19111 and *Salmonella* (*S.*) *enteritidis* ATCC13076 were used for control testing in culturing and molecular methods. All the control strains were received from the Food Microbiology Laboratory of Food Engineering Department located in the Technocenter facilities of İstanbul Aydın University, İstanbul, Turkey.

E. coli O157:H7 25922 was cultured on Tryptone Bile X-glucuronide (TBX) agar (Merck, Germany), *S. aureus* on Baird Parker (BPA) agar (Merck), *L. monocytogenes* on PALCAM agar (Merck), and *S. enteritidis* on Xylose Lysine Deoxycholate (XLD) agar (Merck) under the required conditions according to the manufacturer's instructions. The pure isolates were stored in Tryptic Soy Broth (LABM, UK) containing 10% glycerol at -20°C.

Artificial spiking study

UHT milk was obtained from a foodchain market, and tested for the absence of *E. coli* (Ec), *S. aureus* (Sta), *L. monocytogenes* (Lm) and *S. enteritis* (Sal) by conventional testing methods.

10 ml of UHT milk was transferred to 90 ml of buffered peptone water (BPW) (Oxoid, UK) in a Stomacher filter-bag (Interscience, France). Five suspensions were prepared. So that, four of them were individually spiked with one colony from each Ec, Sta, Lm and Sal by a swab (Adeka, Turkey). The fifth one was inoculated with Ec, Sta, Lm and Sal at all hands. Five uninoculated suspensions were also used as negative controls. After spiking, all the suspensions were homogenized for 2 minutes using a homogenizator (AES Laboratoire-Chemunex, France). Finally, the suspensions were allowed for aerobic incubation for 18-24 hours at 37°C.

Preparation of serial 10-fold dilutions

To determine the sensitivity of *real-time* PCR fourplex assay, 10-fold dilutions were serially performed in 0.85% $NaCl_2$ physiological saline solution for the pre-enriched suspensions. The uninoculated ones were not serially diluted, but microbiologically cultured as negative control. 10 µl of each these serial dilutions was transferred by streaking onto Plate Count (PC) agar plates (Merck) for pre-enrichment. After that, the plates were exposed to aerobic incubation at 30°C for 72 hours. The suspensions were stored in 4-6 °C untill incubation was finalized. After the incubation, the PC agar plates expressing a viable count of 1-10 CFU/ml, 10-100 CFU/ml and 100-1000 CFU/ml were selected for further molecular studies.

Isolation of bacterial DNA

The inoculated pre-enriched suspensions expressing a viable count of 1-10 CFU/ml, 10-100 CFU/ml and 100-1000 CFU/ml in the plates, including the uninoculated ones were used for isolation of bacterial DNA from Ec, Sta, Lm, Sal and a cocktail of these four variants. The extraction of DNA was performed in 10 µl of each of the pre-enriched suspensions by following GENESpin DNA isolation kit (Eurofins, Germany) procedures. Then, the eluted DNA was kept at 4°C for direct use or at −20°C for further processing.

One-step real-time PCR assay

The extracted DNAs were amplified in Stratagene Mx3000P *real-time* PCR (Agilent, Turkey) according to the instructions by a one-step *real-time* PCR assay, PowerChek™ Pathogen 4-plex (Ec, Sta, Lm, Sal) detection kit (Kogene Biotech, South Korea).

Detection limit of the real-time PCR fourplex assay

The detection limit of the fourplex assay was determined by serial dilutions of the variants expressing the concentrations of 1-10 CFU/ml, 10-100 CFU/ml and 100-1000 CFU/ml. A 5 µl aliquot of DNA was mixed with 10 µl of 2x *real-time* PCR master mix, 4 µl of primer/prob mix, and 1 µl of ultra-distilled water, resulting in a 20 µl of total PCR mix. ROX fluorescence

was selected for the target gene VT2 in Ec, FAM for femA in Sta, Cy5 for prfA in Lm, and HEX (VIC) for invA in Sal, respectively. Thermal processing parameters were adjusted as 1 cycle at 95°C for 10 minutes and 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. For each the target bacterial DNAs, the kit's positive control DNA, ultra-distilled water as negative control, and a master mix blank control were all included in the plate. Each measurement was performed in duplicate. Threshold cycle (CT) of the assay Ct ≤ 40 was accepted to be positive in Ec, Sta, Lm, Sal according to the kit instructions.

3. Results

This study evaluated the performance of a *real-time* PCR fourplex assay for simultaneoulsy detecting Ec, Sta, Lm, Sal variants which were artificially spiked to UHT milk within the densities expressed as 1-10 CFU/ml, 10-100 CFU/ml and 100-1000 CFU/ml. The serially

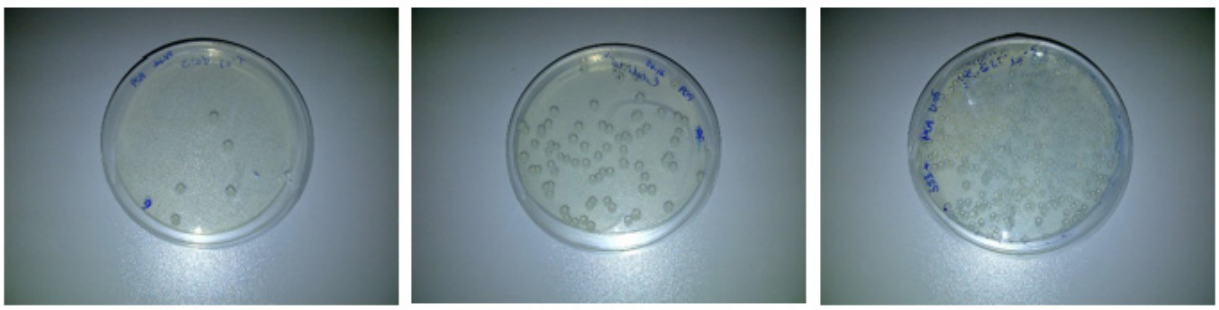

Figure 1. *E. Coli* (EC) PC agar serially diluted cultures

Figure 2. *S. aureus* (Sta) PC agar serially diluted cultures

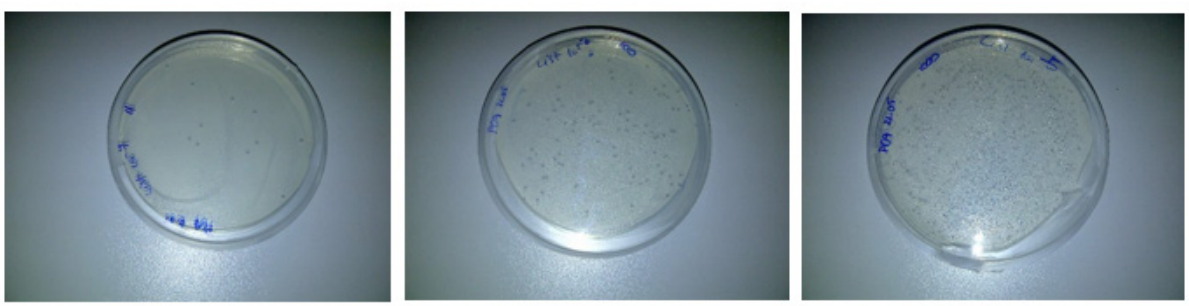

Figure 3. *L. monocytogenes* (Lm) PC agar serially diluted cultures

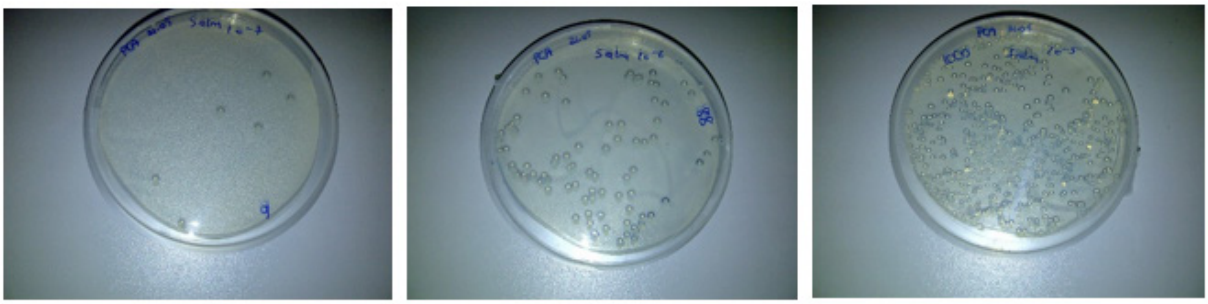

Figure 4. *S. enteritis* (Sal) PC agar serially diluted cultures

diluted cultures from UHT milk were given in Figure 1, Figure 2, Figure 3 and Figure 4.

The detection limit of PowerChek™ Pathogen 4-plex detection kit were tested in five artificially spiked suspensions with different concentrations. Among the suspensions, 4 were individually inoculated with Ec, Sta, Lm and Sal while the fifth suspension was a cocktail harboring all of the four variants. The kit's positive control DNA, ultra-distilled water as negative control, and a master mix blank control were run properly. None of the variant was detected in the uninoculated suspensions.

The multiplex assay did not detect Ec alone within any concentration. Sta and Sal were individually positive for 10-100 CFU/ml and 100-1000 CFU/ml while Lm was determined in 100-1000 CFU/ml only. In the cocktail, PowerChek™ Pathogen 4-plex detection kit could not simultaneoly find the variants in any concentration. The best performance was obtained as triplex for Sta, Lm and Sal in the

Table 1. *real-time PCR screening results*

Species		Density intervals (CFU/ml) and Ct-values							
		Ct	0	Ct	1-10	Ct	10-100	Ct	100-1000
Ec		-	no Ct	-	no Ct	-	no Ct	-	no Ct
Sta		-	no Ct	-	no Ct	+	32.85	+	30.66
Lm		-	no Ct	-	no Ct	-	no Ct	+	37.54
Sal		-	no Ct	-	no Ct	+	34,19	+	33,53
Cocktail	**Ec**	-	no Ct	+	37,64	-	no Ct	-	no Ct
	Sta	-	no Ct	-	no Ct	+	33,65	+	36,53
	Lm	-	no Ct	-	no Ct	+	36,52	-	no Ct
	Sal	-	no Ct	-	no Ct	+	34,42	+	36,59

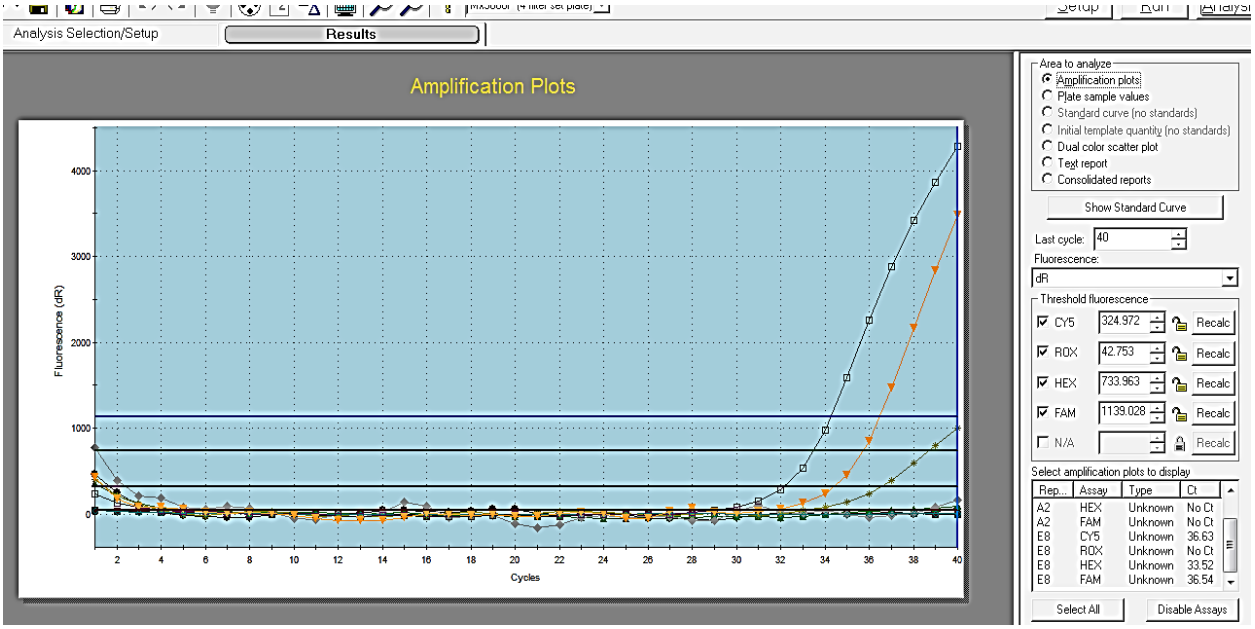

Figure 5. *Amplification plots of Sta, Lm and Sal positive in the cocktail at 10-100 CFU/ml concentration.*

density of 10-100 CFU/ml.

The results revealed that the tested multiplex assay exhibited its best simultaneous performance for a triplex detection, not fourplex. The screening results and the amplification plots positive for Sta, Lm and Sal in a cocktail with density of 10-100 CFU/ml were presented in Table 1 and Figure 5, respectively.

4. Discussion

The *real-time* PCR, with its combination of speed, sensitivity, and specificity in a homogeneous assay, enables us to detect minute amounts of nucleic acids in a wide range of samples in molecular diagnostics, life sciences, agriculture, food, and medicine [15]. However, this technology's popularity is troubled by remarkable technical limitations, including lack of consensus on how to conduct a real-time PCR test; preparation and nucleic acid quality, leading to variable results; poor choice of primers and probes, resulting in inefficient assay performance; contamination; and inappropriate information, delivering misleading results [16,17].

Our study was designed to introduce a *real-time* PCR fourplex molecular biological assay for simultaneous detection and identification of a collection of Ec, Sta, Lm, Sal present in UHT milk. The milk was selected due to the survival of some important pathogens such as *L. monocytogenes* even after post-sterilization, leading to recontamination of dairy products [18].

In the same manner, the literature provided similar studies based on a real-time PCR multiplex assays assay combined with an enrichment step and DNA isolation for *Salmonella* spp., *L. monocytogenes* and *E. coli* O157:H7 [19], for *Salmonella* spp., S. aureus, and *L. monocytogenes* [20], and fourplex assay for *E. coli* O157:H7, *Salmonella* spp., S. aureus, and *L. monocytogenes* [21].

A *real-time* PCR assay does not actually require pre-enrichment in a conventional enrichment media. However, the growth of bacteria in a cultured-based media positively affects the performance of a real-time PCR assay according to the previously conducted

studies [22]. Therefore, our UHT milk was enriched in buffered peptone water (BPW) in order to increase the fourplex kit's sensitivity. Our results indicated that the new assay could not simultaneously provide 100% good sensitivity corresponding to 1-10 CFU/ml, 10-100 CFU/ml and 100-1000 CFU/ml for each Ec, Sta, Lm, Sal and a collection of these four species. It could yield a triplex sensitivity for Sta, Lm, Sal in 10-100 CFU/ml only.

From the technical viewpoint, detecting and differentiating multiplex organisms is strongly dependent on the sufficient concentrations of target regions in the individual species [23], an enrichment in conventional culturing media might not be sufficient to produce adequate numbers of bacterial cells required for a positive real-time PCR result [24].

For instance, *E. coli* O157:H7 contamination levels in milk and milk products were given as <1 CFU/ml [25]. Our study did not correctly identify Ec even in the uninoculated suspension. In this case, poor sensitivity might be arised from insufficient inoculation level of bacteria and limited sets of primers and probs used for this study [26].

In this study, Ct values (33.65 to 36,52) for the fourplex assay with a collection of Sta, Lm, Sal at a concentration of 10-100 CFU/ml were recorded higher than the assay with a pure cultured strains (32.85 to 34.19). Higher Ct values in the multiplex assay might be explained by limited growth of the bacterial strains during pre-enrichment in BPW [25], and some of the major problems associated with matrix characteristics of milk such as fat, protein, calcium, chelators, and dead cells [27]. Thus, our multiplex assay could not offer the possibility of screening of different target genes belonging to four different bacteria.

Our multiplex assay used different dyes to normalize fluorescent signals and fluctuations in fluorescence that were not PCR-based. In this way, flourescent signal of these dyes can be easily distinguished from each other. In this study, our multiplex assay contained ROX dye for Ec [28], FAM for Sta [29], CY5 for Lm to differentiate gene expression in which both control and experimental samples are hybridized to the same array [30], and HEX used for Sal [31], respectively.

5. Conclusions

The fourplex assay we used did not allow us to simultaneously detect and differentiate of the target bacterial species in one step, except for a triplex performance for Sta, Lm, and Sal at a concentration of 10-100 CFU/ml. This situation might be arised from remarkable technical deficiencies, including inadequate enrichment before PCR application, poor inoculation level of bacteria, insufficient DNA isolation, suitability of real-time PCR device for ready-to-use multiplex kit, and some inhibitors associated with matrix characteristics of milk. To conclude, simultaneous detection and differentiation of multiplex bacteria based on real-time PCR platform in used this study are not yet optimized, and still evolving due to some technical limitations.

Conflicts of Interest

The authors declare that there is no conflicts of interest.

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