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Published Version

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Dadkhah, H., Bassami, M. R., Hashemi, S., Shahraz, F., Hosseini, H., Karatzas, K. A. G. and Khaksar, R. (2012) Evaluation and comparison of SYBR Green I Real-Time PCR and TaqMan Real-Time PCR methods for quantitative assay of Listeria monocytogenes in nutrient broth and milk. African Journal of Microbiology Research, 6 (9). pp. 1908-1917. ISSN 1996-0808 doi: 10.5897/AJMR11.889 Available at https://centaur.reading.ac.uk/28742/

To link to this article DOI: http://dx.doi.org/10.5897/AJMR11.889

Publisher: Academic Journals

African Journal of Microbiology Research Vol. 6(9), pp. 1908-1917, 9 March, 2012
Available online at http://www.academicjournals.org/AJMR
DOI: 10.5897/AJMR11.889
ISSN 1996-0808 ©2012 Academic Journals

Full Length Research Paper

Evaluation and comparison of SYBR Green I Real-Time PCR and TaqMan Real-Time PCR methods for quantitative assay of *Listeria monocytogenes* in nutrient broth and milk

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Accepted 27 December, 2011

Specific traditional plate count method and real-time PCR systems based on SYBR Green I and TaqMan technologies using a specific primer pair and probe for amplification of *iap*-gene were used for quantitative assay of *Listeria monocytogenes* in seven decimal serial dilution series of nutrient broth and milk samples containing 1.58 to 1.58×10^7 cfu /ml and the real-time PCR methods were compared with the plate count method with respect to accuracy and sensitivity. In this study, the plate count method was performed using surface-plating of 0.1 ml of each sample on Palcam Agar. The lowest detectable level for this method was 1.58×10 cfu/ml for both nutrient broth and milk samples. Using purified DNA as a template for generation of standard curves, as few as four copies of the *iap*-gene could be detected per reaction with both real-time PCR assays, indicating that they were highly sensitive. When these real-time PCR assays were applied to quantification of *L. monocytogenes* in decimal serial dilution series of nutrient broth and milk samples, 3.16×10 to 3.16×10^5 copies per reaction (equals to 1.58×10^3 to 1.58×10^7 cfu/ml *L. monocytogenes*) were detectable. As logarithmic cycles, for Plate Count and both molecular assays, the quantitative results of the detectable steps were similar to the inoculation levels.

Key words: *Listeria monocytogenes,* real-time PCR, milk, pathogen.

INTRODUCTION

Listeria monocytogenes is a Gram-positive, anaerobic and facultative intracellular bacterium that is recognized worldwide as one of the most important food-borne pathogens of concern for the food industries (Gray et al., 2004; Nakamura et al., 2004; Rodríguez-Lázaro et al., 2004b; Cocolin et al., 2005; Berrada et al., 2006; Rossmanith et al., 2006; Zhang et al., 2007; Rantsiou et al., 2008). This micro-organism has been responsible for listeriosis outbreaks in the past years (Amagliani et al., 2004; Cocolin et al., 2005; Ueda et al., 2005; Kim et al., 2006) and can be isolated from foods of animal origin such as milk and milk products (Nogva et al., 2000; Kaclíková et al., 2004; Makino et al., 2005; Kalorey et al., 2008; Rantsiou et al., 2008). The presence of *L. monocytogenes* in milk-based products can be related to raw milk contamination or to post-pasteurization contamination (Carminati et al., 2004). Furthermore,

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several outbreaks of listeriosis have been proven to be associated with the consumption of milk and are a great concern for the dairy industry (Amagliani et al., 2004). Contamination of milk with this pathogen therefore poses a great health risk to humans.

The detection methods for food borne pathogens generally involve colony isolation on selective media and the use of biochemical tests and serotyping with antibodies against specific bacterial antigens (Aslam et al., 2003; Flekna et al., 2007; Badosa et al., 2009; D'Urso et al., 2009). These conventional methods are laborious and time consuming, and several days are therefore required before results are obtained (Amita et al ., 2002; Cocolin et al ., 2002; Berrada et al ., 2006; Rodríguez-Lázaro and Hernández , 2006; Oravcová et al ., 2007; Reichert-Schwillinsky et al., 2009). Thus, the development of rapid, sensitive and species-specific methods for the detection and quantification of *L. monocytogenes* in milk are required to overcome these limitations**.**

In the last 10 years, Real-Time PCR systems based on SYBR Green I and TaqMan technologies have increasingly been used for accurate and reliable detection and quantification of various food-borne pathogens (Rodríguez-Lázaro et al., 2004a; Rawsthorne and Phister, 2006; Skånseng et al., 2006; Enokimoto et al., 2007; Malorny et al., 2007), including *L. monocytogenes* in contaminated samples along the food production chain. Compared to conventional PCR-based methods, Real-Time PCR technologies involve a lower risk of cross-contamination because the presence of the target sequence(s) in the sample is indicated by an increase in fluorescence signal, and no post-PCR processing of the sample is required (Rodríguez-Lázaro et al., 2004c; Rossmanith et al., 2006). The detection of *Listeria monocytogenes* was accomplished by Real-Time PCR methods directed to the *ssrA* (O' Grady et al., 2008), the *hly* (Norton et al., 2000; Rodríguez-Lázaro et al., 2005), the *actA* (Norton et al., 2001; Oravcová et al., 2006), the *prfA* (Rossmanith et al., 2006), the *iap* (Comi et al., 1997; Hein et al., 2001) and the *inl* (Almeida and Almeida, 2000; Pangallo et al., 2001) genes. Each of these methods is rapid, sensitive and has proven to be specific, but most of them have not yet been compared to the Plate Count method with respect to sensitivity, precision (determined by the standard deviation among replicates of one sample) and accuracy.

Moreover, in Real-Time PCR-based detection systems, amplification efficiencies can be different from sample to sample (Klerks et al., 2004) and application of these systems for direct quantification of pathogens present in foods has been limited by the complex composition of the starting materials which contain inhibitors for PCR amplification. For example, when PCR was applied to milk samples, it's sensitivity was low when compared to that of bacterial cultures (Amagliani et al., 2004), particularly at low concentrations of the pathogen (Amagliani et al., 2004).

The aim of this study was to evaluate and compare the Plate Count method against a SYBR Green I and TaqMan-based Real-Time PCR method for quantitative assay of *L. monocytogenes* in artificially contaminated nutrient broth and milk samples. The applicability of both methods was determined with respect to accuracy and sensitivity.

MATERIALS AND METHODS

Bacterial strain, medium, and culture conditions

L. monocytogenes ATCC 19115 was obtained from the Iranian Research Organization for Science and Technology (IROST) and used in all molecular and culture methods. The strain was grown overnight at 37°C in Nutrient Broth (Merck, Germany). Subsequently, using a culture containing 1.58×10^8 cfu *L. monocytogenes* per ml, decimal serial dilutions containing 1.58 to 1.58×10^7 cfu/ml were prepared in the nutrient broth itself. The cell concentrations of these decimal serial dilutions were estimated in triplicate by surface-plating of 0.1 ml of each sample on Palcam agar (Merck, KGaA, Darmstadt, Germany) following incubation at 37°C for 24 h. For Real-Time PCR quantification, the serial dilutions were directly subjected to DNA extraction. Moreover, *E. coli* O157:H7 ATCC 43895 (IROST) was used as negative control in molecular methods.

Artificially contaminated milk samples

UHT-treated milk (2.5% fat) was purchased from a local supermarket. Subsequently, decimal serial dilutions of milk containing 1.58 to 1.58×10^7 cfu *L. monocytogenes* per ml were prepared. These serial dilutions were prepared by the addition of 1 ml of each of the decimal serial dilutions of nutrient broth containing 1.58×10 to 1.58×10^8 cfu *L. monocytogenes* per ml to 9 ml of UHT-treated milk. Similar to contaminated nutrient broth samples, the bacterial levels in each contaminated milk sample were determined in triplicate by surface-plating of 0.1 ml of each sample on PALCAM Agar (Merck, Germany) followed by incubation at 37°C for 24 h. After the artificial contamination of the milk samples, for molecular quantification of the bacterial numbers, the serial dilutions were directly subjected to DNA extraction.

DNA extraction

DNA extraction from both cultures and contaminated milk samples was performed as follows. Five hundred µl of TE buffer (10mM Tris, 1 mM EDTA, pH 8.0) was added to 500µl of each sample, incubated at 85-90°C for 15 min and immediately placed at -20°C for 20 min. Once samples were removed from the freezer, 100µl (20 mg/ml) lysozyme was added and incubated at 37°C for 2 hrs. Subsequently, 75µl proteinase K (20 mg/ml) was added followed by incubation at 60-65°C for 20 min.

Then, equal parts of 470µl of lysis buffer, isopropanol and each sample were transferred to a 1.5 ml sterile filter tube and centrifuged (10,000 × g for 1 min). The supernatant was washed once with 750 µl of wash buffer I and twice with 750 µl of wash buffer II (Roche Molecular Biochemicals, Indianapolis, IN, USA). Following centrifugation at 14, 000 ×*g* for 3 min, 50 µl of pre-warm elution buffer (Roche Molecular Biochemicals, Indianapolis, IN, USA) was added. After incubation for 5 min. at room temperature and final centrifugation step (8000 ×*g* for 1 min), 5µl of the final elute was used for quantitative Real-Time PCR reactions with respect to factor of 50.

Table 1. Sequences of the oligonucleotides and their locations in the *iap*-gene of *L. monocytogenes*.

Primer name	Sequence (5'_ to 3')	Location	Concentration in the PCR mix (nM)	Reference
LIM 2 (forward primer)	CTA AAG CGG GAA TCT CCC TT	1214–1233	250	Hein et al., 2001
LIMRE (reverse primer)	CCA TTG TCT TGC GCG TTA AT	1369–1388	250	Hein et al., 2001
LMS 2 (probe)	FAM-CTT CTG GCG CAC AAT ACG CTA GCA CT-TAMRA	1241–1266	250	Hein et al., 2001

Primers and probe

Detection of *L. monocytogenes* was based on the amplification of *iap* gene with the use of PCR-primers and probe (Table 1) as previously described by Hein et al. (2001). Quantification using SYBR Green I required the use of the LIM 2 and LIMRE primers, while the use of TaqMan required the use of the previous primer pair in combination with LMS 2 probe. The specific TaqMan probe was labelled at the 5′ and the 3′ end with a 6-carboxy-fluorescein group (FAM) and 6-carboxytetramethyl-rhodamine (TAMRA), respectively. Primers and probe were synthesized by Metabion (Munich, Germany).

Real-Time PCR amplification conditions

For generation of standard curves and quantitative assays, amplifications were carried out with 5µl of elute, extracted as described above, in a final volume of 20µl using the MiniOpticon Real-Time PCR Detection System (BioRad, Hercules, CA, USA).

For detection with SYBR Green I, following the addition of 5µl of elute in the tube, 4.0µl of 5×Eva Green (BioAtlas, Estonia and Finland) was added. $MgCl_2$ was also added up to a concentration of 5mM while primers were added up to a final concentration of 250nM. Based on optimization of annealing temperature, the cycling conditions included an initial denaturation at 95°C for 15 min followed by 45 cycles of 15 s denaturation at 95°C, 30 s annealing at 54°C and 30 s extension at 72°C. The thermocycling program was followed by a melting program of 95°C for 1 min. (denaturation), 45°C for 30 s (annealing), and then a transition from 45 to 95°C with a rate of 0.1°C/s. Monitoring of fluorescence was performed at regular intervals during the annealing step and continuously throughout the melting phase.

For detection with TaqMan, following the addition 5µl elute of extracted DNA to the tube 4.0 µl of 5×AmpliTaq (BioAtlas, Estonia and Finland) was added. $MgCl_2$ was also added up to a concentration of 5mM while primers and probe were added at a final concentration of 250 nM. The amplification proceeded with an initial step of 95°C for 15 min followed by 45 cycles of 95°C for 15 s and 57°C for 1 min. (temperature optimized through a gradient protocol). All molecular assays were performed in triplicate for each sample.

Generation of standard curves

DNA extraction from *L. monocytogenes* used for construction of standard curves and sensitivity testing was carried out using a High Pure PCR template preparation kit (Roche Molecular Biochemicals, Indianapolis, IN, USA) as described by the manufacturer.

The concentration of the extracted DNA was measured by NanoDrop ND-1000 Spectrophotometer (BioPhotometer, Eppendorf, Hamburg, Germany). The correlation of the signal detected by the increase of the amplicons during the Real-Time PCR to the original number of copies of the *iap* gene was based on the fact that only a single copy of the *iap*-gene is contained in the genome (Hein et al., 2001) and that 1ng of listerial DNA equals 3.1×10^5 copies of the whole genome (Rossmanith et al., 2006).

RESULTS

Standard curves and linear range of quantification

The concentration of the extracted DNA used for construction of standard curves was 0.258 µg/µl, which corresponds to 8×10^7 copies of the *iap*-gene. To estimate the detection limits of the Real-Time PCR assays, 6 serial decimal dilutions of the extracted DNA containing 4×10^5 to 4 copies of the *iap*-gene in the 5 µl used for each reaction were prepared. Both Real-Time PCR methods were very sensitive and they were able to detect as few as four copies per reaction. Standard curves of the logarithm of the quantity of the template against the C_T were constructed (Figure 1) and their slopes with SYBR Green I and TaqMan were -3.395 and -3.230 respectively while their coefficients of correlation (r^2) were 0.999 and 1, respectively, indicating that the Real-Time PCR systems were highly linear over a range of five logarithms.

The specificity of both Real-Time PCR assays was assessed by examining 10-fold serial dilutions of whole genomic DNA of *L. monocytogenes*. For both molecular assays, agarose gel (1.5%) electrophoresis of the amplification products of these dilution series were visualized under UV light and all displayed the expected band of 175 bp. Moreover, no amplified DNA fragments were observed when the primers and probe from two negative controls (a different organism's DNA and a control with no DNA template) (Figure 2).

Quantitative detection of artificially contaminated nutrient broth and milk samples using plate count method

Nutrient broth and milk samples artificially contaminated by the serial decimal dilution series of *L. monocytogenes* ranging from 1.58 to 1.58×10^7 cfu/ml were analyzed in triplicate by surface-plating on PALCAM Agar (Table 2). The plate count method was able to detect the numbers estimated according to the inoculation levels. The lowest detectable level of *L. monocytogenes* was 1.58×10 cfu

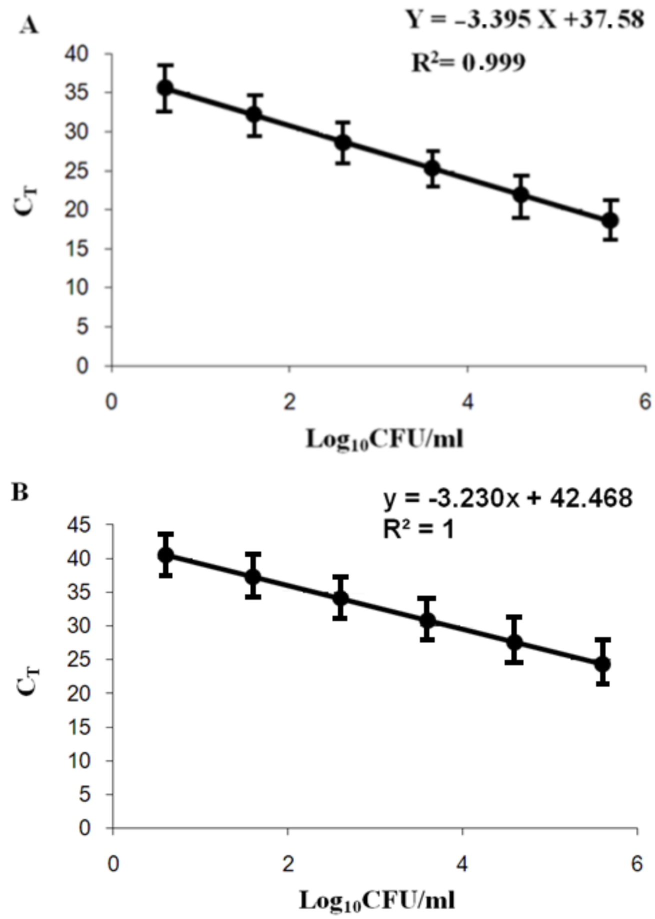

Figure 1. Standard curves of the SYBR-Green I Real-Time PCR (A) and TaqMan Real-Time PCR (B) performed using as template the 10-fold serial dilutions of genomic DNA extracted from *L. monocytogenes*.

per ml nutrient broth and milk samples. The mean of the three replicates measurements of the lowest detectable level by Plate Count method was 1.33×10 and 2.00×10 cfu per ml nutrient broth and milk samples, respectively.

Calculation of the copy number of the *iap*-gene in artificially contaminated nutrient broth and milk samples using SYBR Green I Real-Time PCR

Table 3 shows the results of SYBR Green I detection of artificially contaminated nutrient broth and milk samples. For both inoculated nutrient broth and milk samples, five decimal dilution series of the extracted genomic DNA, ranged from 3.16×10 to 3.16×10^5 copies per reaction were detectable. These five dilutions were extracted from inoculated nutrient broth and milk samples containing 1.58×10^3 to 1.58×10^7 cfu/ml *L. monocytogenes*. Therefore, using SYBR Green I molecular assay, the lowest detectable level of genomic DNA extracted from nutrient broth and milk samples was 3.16×10 copies per reaction, which equals1.58×10^3 cfu *L. monocytogenes* per ml. The mean of the three replicates measurements of this level by SYBR Green I system was 2.4×10 copies per reaction (equal to 1.20×10^3 cfu/ml) for nutrient broth samples and 4.22×10 copies per reaction (equal to 2.11×10^3 cfu/ml) for milk samples. Moreover, for both nutrient broth and milk samples the five detectable dilutions showed the numbers estimated according to logarithmic decades of the inoculation levels.

Calculation of the copy number of the *iap*-gene in artificially contaminated nutrient broth and milk samples using TaqMan Real-Time PCR

Similar to those obtained for SYBR Green I quantitative assay, five decimal dilution series of the extracted genomic DNA, ranging from 3.16×10 to 3.16×10^5 copies per reaction, were detectable using TaqMan molecular assay (Table 4). As SYBR Green I system, these five dilutions were extracted from inoculated nutrient broth

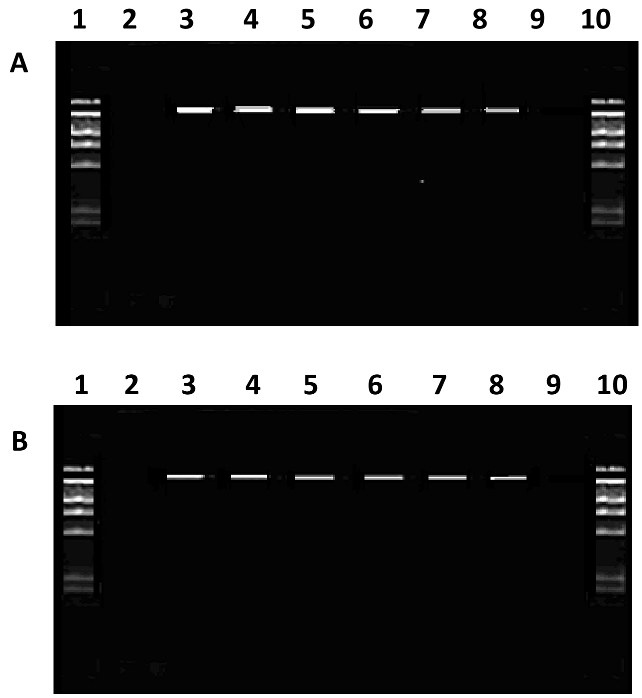

Figure 2. Analytical sensitivity results of amplification products of the SYBR-Green I Real-Time PCR (A) and TaqMan Real-Time PCR (B). Lane 1: 100-bp ladder; lane 2: control with no DNA template; lanes 3–8: ten-fold dilution series of whole genomic DNA (ranging from 4 –4 $\times10^5$ copies of the iap gene/PCR reaction); lane 9: different organism's DNA; lane10: 100-bp ladder.

and milk samples containing 1.58×10^3 to 1.58×10^7 cfu/ml *L. monocytogenes*. Moreover, the obtained quantitative results of evaluation of these five detectable steps showed the same logarithmic decades of the inoculation levels. The mean of the three replicated measurements of the lowest detectable level (3.16×10 copies per reaction, equal to 1.58×10^3 cfu/ml *L. monocytogenes*) by TaqMan quantitative assay was 2.38×10 copies per reaction (equal to 1.19×10^3 cfu/ml) for nutrient broth samples and 2.86×10 copies per reaction (equal to 1.43×10^3 cfu/ml) for milk samples.

DISCUSSION

PCR is a technique that enables the detection of the presence of food-borne pathogens. Several papers have focused on its use for the detection and quantification of *L. monocytogenes*. However, few studies are available which compare PCR-based assays with traditional Plate Count methods with respect to their accuracy and sensitivity. Hence, in this study, using a specific primer pair and probe, targeting the *iap*-gene, two Real-Time PCR methods (SYBR Green I and TaqMan Real-Time PCR assays) were used for specific quantification of decimal serial dilutions of *L. monocytogenes* in artificially contaminated nutrient broth and milk samples. Finally, after evaluation of the obtained results of these quantitative assays the results were compared with the obtained results of traditional Plate Count method using PALCAM Agar, with respect to accuracy and sensitivity.

The Plate Count method showed quantitative results of expected logarithmic decades, indicating the accuracy of this method for quantification detection of artificially contaminated nutrient broth and milk samples. Because of the plating of 0.1 ml of each sample the samples inoculated by cfu<10 per ml sample were not detectable. Using purified DNA dilutions as a template, as few as four *iap*-gene copy numbers per reaction were detected by the both molecular assays, which was two copy numbers lower than that reported by Hein et al. (2001).

The numbers of cells estimated with both Real-Time PCR assays were similar to those obtained by the plate count method, indicating the accuracy of these methods for the quantification of *L. monocytogenes* present in artificially contaminated nutrient broth and milk samples. The dilution coefficient of 50 in DNA extraction step made the samples inoculated by cfu<50 per ml not detectable. However, because of PCR inhibitors and other factors, the samples inoculated by 1.58 $\times10^2$ cfu/ml were not detectable. Quantitative results of SYBR Green I and TaqMan systems indicate the seam sensitivity and

Table 2. Quantitative detection of 10-fold serial dilutions of *L. monocytogenes* in artificially contaminated nutrient broth and milk samples using Plate Count method. The samples were evaluated in triplicate.

Sample	Plate count method (CFU/ml)×10					
	Nutrient broth	Mean	SD	Milk	Mean	SD
1	1.83×10^7 1.29×10^7 1.86×10^7	1.66×10^7	0.32×10^7	1.96×10^7 2.22×10^7 1.85×10^7	2.01×10^7	0.19×10^7
2	1.15×10^6 1.06×10^6 1.24×10^6	1.15×10^6	0.09×10^6	2.84×10^6 1.28×10^6 1.67×10^6	1.93×10^6	0.81×10^6
3	2.52×10^5 2.00×10^5 1.81×105	2.11×10^5	0.36×10^5	3.21×10^5 1.05×10^5 3.37×10^5	2.54×10^5	1.29×10^5
4	1.16×10^4 3.04×10^4 3.33×10^4	2.51×10^4	1.17×10^4	1.31×10^4 1.91×10^4 0.98×10^4	1.40×10^4	0.47×10^4
5	2.44×10^3 1.27×10^3 3.00×10^3	2.24×10^3	0.88×10^3	1.59×10^3 1.50×10^3 2.19×10^3	1.76×10^3	0.37×10^3
6	1.90×10^2 1.20×10^2 1.70×10^2	1.60×10^2	0.36×10^2	1×10^2 1.10×10^2 1.50×10^2	1.20×10^2	0.26×10^2
7	1×10 2×10 1×10	1.33×10	0.57×10	4×10 1×10 1×10	2.00×10	1.73×10
8	ND ND ND	ND	-	ND ND ND	0.33	-

ND: Not detectable.

accuracy of these methods. However, due to the high cost of probe synthesis, the TaqMan Real-Time PCR system cannot necessarily be used for quantification and detection of *L. monocytogenes*.

Generally, the potential of the investigated techniques for quantification of *L. monocytogenes* are influenced by a number of factors that are not completely understood. Depending on the distribution of the microorganisms in the sample and the presence of the aggregated bacterial cells, different quantitative results may be obtained by the Plate Count method. In addition, the sample preparation step prior to detection is of key importance when applying Plate Count or molecular systems to detection and quantification of microorganisms in foods. If the levels of contamination are low and small volumes of the sample are used in the plating or extraction steps, or a high dilution of coefficients is used in these steps, the bacterial cells might not be detected and quantified. Sometimes dead and viable but non-culturable bacteria cells that are unable to grow on solid agar media, and therefore cannot be quantified by the Plate Count method, are also present. However, these cells can be detected and quantified using the Real-Time PCR methods. This poses a serious problem since the main purpose of the detection of *L. monocytogenes* in foods is the food safety and the protection of the consumer from a potentially hazardous bacterium, and it is obvious that dead or viable but non-culturable cells do not pose any threat to the consumer. Therefore, it is possible that the implementation of these Real-Time PCR methods on their own might lead to high fines and false rejection of absolutely safe products, which can lead to unfair treatment of food

Table 3. Quantitative detection of 10-fold serial dilutions of *iap* gene copy numbers in artificially contaminated nutrient broth and milk samples using SYBR-Green I Real-Time PCR assay. Dependently DNA extraction was used for the three replicate of each sample.

Sample	SYBR-Green I Real-Time PCR assay					
	Nutrient broth			Milk		
	Copy number/ml×50	Mean	SD	Copy number/ml×50	Mean	SD
1	$2.00×10^7$ $1.00×10^7$ $1.00×10^7$	$1.33×10^7$	$0.57×10^7$	$1.06×10^7$ $1.06×10^7$ $1.22×10^7$	$1.11×10^7$	$0.09×10^7$
2	$1.01×10^6$ $1.63×10^6$ $1.11×10^6$	$1.25×10^6$	$0.33×10^6$	$3.60×10^6$ $2.39×10^6$ $2.21×10^6$	$2.73×10^6$	$0.75×10^6$
3	$3.00×10^5$ $3.00×10^5$ $3.00×10^5$	$3.00×10^5$	$0.00×10^5$	$1.50×10^5$ $1.50×10^5$ $1.50×10^5$	$1.50×10^5$	$0.00×10^5$
4	$0.80×10^4$ $0.71×10^4$ $1.20×10^4$	$0.90×10^4$	$0.26×10^4$	$1.60×10^4$ $1.69×10^4$ $1.78×10^4$	$1.69×10^4$	$0.09×10^4$
5	$0.91×10^3$ $1.69×10^3$ $1.00×10^3$	$1.20×10^3$	$0.42×10^3$	$2.23×10^3$ $1.99×10^3$ $2.11×10^3$	$2.11×10^3$	$0.12×10^3$
6	ND ND ND	ND	-	ND ND ND	ND	-
7	ND ND ND	ND	-	ND ND ND	ND	-
8	ND ND ND	ND	-	ND ND ND	ND	-

ND: Not detectable.

producers and high economic costs. In PCR-based assays the efficiency of amplification is influenced by the presence of "PCR inhibitors". These matrix-associated interfering substances may be attributed to the food sample itself or the culture medium. Many of "PCR inhibitors" have not been identified but some are known substances. These substances include chelators of cations and substances that degrade or bind the polymerase or the DNA template. For example, milk contains high levels of cations (Ca^{++}), nucleases, fatty acids, proteases and DNA. Therefore, an extraction protocol for removing inhibitors similar to the one described here could reduce the concentration of PCR inhibitors, for DNA extraction from milk and nutrient broth samples. In addition, the application of an efficiency test of decimal serial dilutions could show the presence of these inhibitors. If these problems can be overcome, these Real-Time PCR-based assays, due their accuracy and rapidity, offer a good alternative to the Plate Count method.

Detection and quantification of *L. monocytogenes* and other food-borne pathogenic microorganisms in milk as a very important food product have played a significant role in the control and prevention of further human infections. Therefore, numerous studies have been reported in the literature on the development of rapid, specific and sensitive molecular methods based on new target genes and optimized conditions. According to these published

Table 4. Quantitative detection of 10-fold serial dilutions of *iap* gene copy numbers in artificially contaminated nutrient broth and milk samples using TaqMan Real-Time PCR assay. Dependently DNA extraction was used for the three replicate of each sample.

Sample	TaqMan Real-Time PCR assay					
	nutrient broth			Milk		
	Copy number/ml×50	Mean	SD	Copy number/ml×50	Mean	SD
1	$1.59×10^7$ $1.50×10^7$ $1.09×10^7$	$1.40×10^7$	$0.26×10^7$	$2.07×10^7$ $2.07×10^7$ $2.07×10^7$	$2.07×10^7$	$0.00×10^7$
2	$1.00×10^6$ $3.50×10^6$ $1.00×10^6$	$1.83×10^6$	$1.44×10^6$	$0.75×10^6$ $1.13×10^6$ $1.13×10^6$	$1.00×10^6$	$0.21×10^6$
3	$1.63×10^5$ $1.60×10^5$ $0.67×10^5$	$1.30×10^5$	$0.54×10^5$	$1.70×10^5$ $1.65×10^5$ $1.45×10^5$	$1.60×10^5$	$0.13×10^5$
4	$2.00×10^4$ $2.20×10^4$ $2.20×10^4$	$2.13×10^4$	$0.11×10^4$	$1.04×10^4$ $1.04×10^4$ $1.04×10^4$	$1.04×10^4$	$0.00×10^4$
5	$1.19×10^3$ $1.19×10^3$ $1.19×10^3$	$1.19×10^3$	$0.00×10^3$	$1.11×10^3$ $1.91×10^3$ $1.28×10^3$	$1.43×10^3$	$0.42×10^3$
6	ND ND ND	ND	-	ND ND ND	ND	-
7	ND ND ND	ND	-	ND ND ND	ND	-
8	ND ND ND	ND	-	ND ND ND	ND	-

ND: Not detectable.

molecular protocols, development of specific target genes, primer pairs, probe and other conditions seem more than sufficient for specific identification of *L. monocytogenes* in different foods, such as milk and milk products. Therefore, future studies must be focused mainly on efficient DNA extraction from different matrixes, development of new protocols for specific identification of other food-borne pathogenic microorganisms in food products and validation of these alternative methods with respect to sensitivity, specificity and accuracy.

Conclusions

In this study, we evaluated and compared two Real-Time PCR quantitative methods against the Plate Count method for quantification of dilution series of *L. monocytogenes* in two different matrixes. The Real-Time PCR methods showed similar accuracy for quantitative detection of examined samples, but the sensitivity of Plate Count method was two logs lower than the investigated molecular assays. Finally, according to the obtained results and with respect to the advantages of the molecular systems, these assays can be considered a powerful alternative to traditional cultural methods of pathogen quantification in different foods and culture media matrixes.

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