Rapid Quantitative Detection of *Listeria monocytogenes* in Chicken Using Direct and Combined Enrichment/qPCR Method

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ABSTRACT: *Listeria monocytogenes is a species of foodborne pathogen often related with foods, such as poultry, ready-to-eat products, fruits, and vegetables. The culture method is a standard procedure for the detection of bacteria in food products. The real-time quantitative PCR (qPCR) technique can be used for the quantification of foodborne pathogens. The current research was aimed to assess and compare the culture, conventional PCR, and qPCR methods for detecting L. monocytogenes in chicken carcass specimens collected from various slaughterhouses situated in Mashhad in Iran. The TaqMan approach was applied for amplifying a 160 bp fragment corresponding to the prfA gene in the qPCR method. The Limit of Detection (LOD) was obtained at 27 CFU/25 g. Among 100 individual specimens tested, 13 specimens were recognized positive for L. monocytogenes using the culture method, 15 samples by the direct qPCR method, and 17 samples by the enrichment/qPCR method. From the similarity of the results of PCR and qPCR, without the enrichment stage, it can be concluded that the DNA of the bacteria killed in the specimens was due to the presence of previous contamination, which can be determined by determining the number of copies of DNA by qPCR. The severity of contamination indicates the health status of the foodstuff. Our results indicate that qPCR is an effective, quicker, and more sensitive technique for the identification of L. monocytogenes in chicken.*

KEYWORDS: *Listeria monocytogenes; Real-time PCR; prfA; Foodborne pathogen.*

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INTRODUCTION

The *L. monocytogenes* is a major concern for the food industry since it is capable of growing at refrigerator temperatures [1, 28]. Food products, such as meat and poultry foods, are commonly contaminated by *L. monocytogenes*, which has proven itself to be the main source of listeriosis in humans [29]. Several incidences of food and meat contamination have been reported due to *L. monocytogenes*, which have resulted infoodborne disease outbreaks [30, 31].

Listeria monocytogenes is a Gram-positive bacteria and the main source of food-borne illnesses worldwide [1-6]. *Listeria* disease in humans has a low prevalence but mortality rate of about 16%. It also has a high mortality rate among a group of people in the community, including women and children [1, 7]. In 2004, using standard culture and PCR methods in Belgium, the prevalence of *Listeria monocytogenes* in chopped meat was about 42% and in smoked fish was about 33% .Also, in a survey of the 2000-2007 journals in China, the prevalance of *Listeria monocytogenes* in raw meat was reported at about 25% [8, 9].

Culture methods for detecting *L. monocytogenes* in edibles are slow and laborious; besides, they may not be convenient for raw meat and foods that have short shelf-lives [26, 31,33].Since there is "zero tolerance" toward the existence of *Listeria* in certain foods meant for consuming by human in many countries, the food industry needs a fast and sensitive experiment for the routine microbiological analysis of the food items to ensure the their products safety [33, 34]. Molecular methods such as the PCR techniques have been used to recognize the foodborne pathogens. The real-time PCR technique offers higher speed, sensitivity, and reproducibility than other methods. In addition, qPCR decreases the danger of cross- pollution comparing to conventional PCR, and it also determines the quantification [26, 35, 36].

Poultry meat is a usual source of *L. monocytogenes* infection in humans, which leads to sporadic as well as epidemic listeriosis [1]. Major outbreaks of foodborne diseases have been reported on almost every continent in the past decade [1, 6, 10-13]. In Iran,2,250 foodborne disease outbreaks caused by bacterial agents were recorded between 2006–2011 [14]. The gold standard test for detecting *L. monocytogenes* is the culture technique [15, 16]; however, due to its requirement for selective enrichments (24–48 h) and separation on selective media (48h), this method is time-consuming and laborious. To detect *L. monocytogenes* using this method, it requires five days for confirming a negative sample and up to 10 days for positive samples [17-20]. Essentially, the food production industry needs rapid detection approaches for their microbiological quality control programs. Several methods have been improved for this objective, where in most of them involve DNA-based PCR approaches for the detection of the target organisms [21, 22]. In addition, the quantitative detection of bacterial pathogens using the qPCR method has been done in food quality control laboratories.

The objectives of the current research were to: (1) assess and compare the traditional culture method with PCR and qPCR techniques for detection *L. monocytogenes* in chicken specimens, and (2) study the effect of pre-enrichment effect on the detection capacity of the PCR and qPCR techniques.

EXPERIMENTAL SECTION

Bacterial strain growth conditions:

Bacterial strains of *Escherichia coli* ATCC 35218, *L. monocytogenes* ATCC 6744, *L. monocytogenes* ATCC 19114, *Listeria innocua* ATCC 33090, *Listeria ivanovii* ATCC BAA-139, *Salmonella paratyphi A*ATCC9150, *Salmonella enterica* subsp.*enterica* CCM 3807, and *Staphylococcus aureus* ATCC 12600 were grown on Trypticase soy agar (TSA, Merck, Germany) and accompanied with 0.6 % yeast extract. The liquid culture for a single colony was prepared with 10 mL of Trypticase soy broth (TSB, Merck, Germany) and accompanied with 0.6 % yeast extract, which was mixed and incubated with shaking (50 rpm) at 37 °C overnight (16–20 h).

Sample preparation

In total, 100 whole dressed chicken carcasses (1.8-2 kg) were collected from various slaughterhouses in Mashhad, Iran and transported to the laboratory immediately at a controlled temperature of 4 °C. Each carcass was then placed in a sterile sampling bag (50 × 37.5 cm) containing 300 mL of 0.1% peptone water (PW), and shaken by hand for 2 min. The liquid was collected and its centrifugation was performed at 5000 rpm for 15 min (Eppendorf, 5415R) [23, 24]; then the pellet was resuspended in a 10 mL normal Ringer's solution. Afterward, 5 mL resuspension aliquots were frozen with no

enhancement for the analysis using the qPCR (direct detection) method. The remaining resuspension was used for analyzing using the culture method and qPCR after applying an enrichment procedure.

Detection of L. monocytogenes using the standard culture technique

The aliquots were enriched in the Listeria enrichment broth (LEB) (Oxoid, CM0862) and then its incubation was performed at 37 °C for overnight. After incubation, they were analyzed using the culture method as the gold standard test and qPCR as the comparative test. The *L. monocytogenes* was detected after enriching the samples in the LEB incubated at 37 °C for 24 h, and surface plating on Oxford agar (Liofilchem, Italy). Enrichment and direct plating were carried out based on the standard protocol and the characteristic colonies were confirmed by the Microgen® Listeria ID kit (Microgen Bioproducts Ltd, UK).

Genomic DNA extraction

An extraction of genomic DNA from the pure culture of *L. monocytogenes* ATCC 6744 and the chicken rinse sample was performed using the High Pure Template Preparation Kit (Roche, Germany) based on the producer instructions. The DNA concentration was quantified using the "Micro Volume Spectrophotometer" (Nano Drop-ND 1000 Spectrophotometer, USA).

Conventional PCR assay

PCR amplification of *prs* (*Listeria* spp.) and *lmo*1030 (*Listeria monocytogenes*) was performed. The information regarding the forward and reverse primers utilized in current research and the target genes are presented in Table 1. In addition, the specificity for all primer pair has previously been tested elsewhere [2, 11]. The PCR was performed with the Master cycler gradient (Eppendorf AG, 22331, Germany). The reaction was performed for 5 min at 94 °C for the initial period, followed via annealing for 30 s at 60 °C, extension for 30 s at 72 °C, and a final extension period at 72 °C for 5 min (45 cycle). The amplified fragments were isolated using 1.5 % agarose gel electrophoresis (Invitrogen, USA) in 1X Tris-Borate-EDTA (TBE; Invitrogen, USA) and then stained with SYBR Safe DNA gel stain (Invitrogen, USA) for 30 min. The gels were found and the images were obtained using the gel documentation system (Syngene, UK).

The qPCR assay

The qPCR was carried out in a Rotor-Gene Q real-time PCR thermocycler (Qiagen, USA) and the presence and quantitative detection of *L. monocytogenes* was tested in the chicken rinse before and after the enrichment step. This test was done as a comparative test. The DNA extraction was performed as explained in the top section of this study. The primers used for amplifying the transcripts of the *prfA* gene for the qPCR were planned with the Primer Premier software (PREMIER Biosoft Int., USA), which was synthesized by Bioneer (Bioneer Corporation, Daejeon, South Korea). The TaqMan probe was labeled with 6-carboxyfluorescein (FAM, the reporter dye) at the 5′ end and the Black Hole Quencher 1, 3` (BHQ1-3) was used in the real-time PCR assay [25].

The primers and probe sequences utilized for the *prfA* (amplicon size: 160 bp) amplification were as followings: forward primer, 5´-AACTACTGAGCAAGAATC-3´; reverse primer, 5´- CTTTACCATACACATAGG-3´; and probe, 5´-FAM- TTAAAAGTTGACCGCA-BHQ1-3´. In order to determine the specificity for the primers and the probe, qPCR was performed with eight strains including two target strains and six non-target strains (Table 2). The qPCR reactions were carried out in a last volume of 20 µL comprising 10 µL of Amplicon 2x Real IQ-PCR master mix (7 mM MgCl2), 1 µL of the forward primer (10 pmol/µL), 1 µL of the reverse primer (10 pmol/µL), 0.5 µL of the probe (10 pmol/µL), 3 µL of the template DNA (40.8 ng/µL), and 4.5 µL distilled water. The amplification step, following the period for 5 min denaturation at 94 °C, was carried out in 45 cycles for 25 s at 94 °C, and for 20 s 51 °C. The non-specific amplification was determined after the PCR products had been separated on 1.5 % agarose gels [26]. Negative control for amplification was performed with 3 µL of distilled water as a replacement DNA template. The reactions were performed as triplicates. The real-time PCR assays with Cycle Threshold (CT) values ≥ 45 were considered as negative [26].

Standard curves and amplification efficiency

The estimation of the qPCR efficiency was done by incubating a pure culture of *L. monocytogenes* in 10 mL of TSB at 37 °C followed by extracting DNA using High Pure Template Preparation Kit (Roche, Germany). The genomic DNA was determined using Nano-Drop ND-1000

Table 1: Primer pair sequence, target gene for conventional PCR utilized in this study [11].

Species	Gene	Primer Sequences (5'-3')	Product(bp)
Listeria spp.	*prs*	F GCTGAAGAGATTGCGAAAGAAG	370
		R CAAAGAAACCTTGGATTTGCGG	
Listeria monocytogenes	*Lmo1030*	F CTTGTATTCACTTGGATTTGTCTGG	509
		R ACCATCCGCATATCTCAGCCAACT	

Table 2: Specificity of the qPCR assay for different bacterial strains.

Bacterial species	Strain	qPCR results of *PrfA* gene
Listeria monocytogenes	ATCC 19114	+
Listeria monocytogenes	ATCC 6744	+
Listeria innocua	ATCC 33090	-
Listeria ivanovii	ATCC BAA-139	-
Salmonella paratyphi A	ATCC 9150	-
Salmonella entericasubsp. enterica	CCM 3807	-
Staphylococcus aureus	ATCC 12600	-
Escherichia coli	ATCC 35218	-

(Thermo Scientific, USA) and the copy number of the *prf A*gene was computed based on the method developed by *Rossmanith et al.* [25] (Figs. 1, 2).

Evaluation of the limit of detection (LOD) in the chickens using the qPCR method

The growth of *L. monocytogenes* ATCC 6744 was done overnight at temperature of 37 °C in 10 mL of TSB; then tenfold serial dilutions were done to obtain the final concentration. At this stage, the dilutions were achieved using McFarland turbidity standards (10^1 to 10^8 CFU/g). Afterward, 25 g of chicken, which was previously shown to be negative for *L. monocytogenes* presence using the standard protocol and Microgen® Listeria ID kit, was added to 225 mL of peptone water and the contents were inoculated with 10^1, 10^2, and 10^3 CFU/mL concentrations of *L. monocytogenes* ATCC6744. The resultant mixture was then homogenized for 2 min and DNA extraction was performed in the same process as. The plate count tests were performed to estimate the cell numbers of *L. monocytogenes* (CFU/g) [17, 27].

Statistical analysis

In this study, we have used descriptive statistics, such as bar charts and crosstabs, to compare the tests collectively. The Kappa and Fisher's exact tests were used for this purpose, whereas the ROC curve and relative measures were performed for determining the sensitivity and specificity of the test. The significance level of the tests was considered at 5 %. The data was analyzed using the R statistical software.

Sensitivity (%) = 100 * TP / (TP + FN).

Specificity (%) = 100 * TN / (TN + FP).

RESULTS AND DISCUSSION

Specificity of the primers and probe for qPCR

The specificity for the primers and probe was examined by two target strains as the positive controls and six bacterial strains (Table 2) as the negative controls. As shown in Table 2, the qPCR trials did not indicate any of the non-target strains as positive and flagged only for the target strains; this shows the high specificity of the primers and probe.

Quantification assays

In order to establish a standard curve, series of the genomic DNA isolated from *L. monocytogenes* ATCC 6744 were diluted by 10-fold for the efficiency assay of the real-time PCR method; the slope of the linear regression curve was -3.374 and the amplification efficiency was

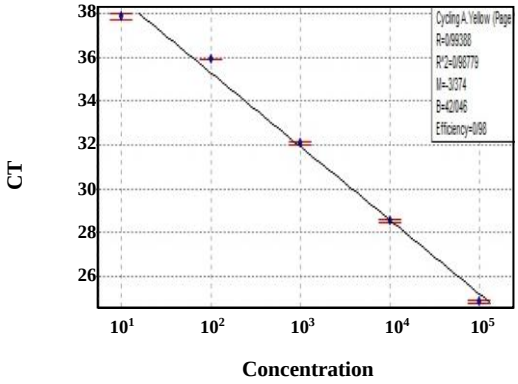

Fig. 1: Standard curves created using qPCR amplification of serially diluted purified DNA showed as log of genome equivalents/reaction.

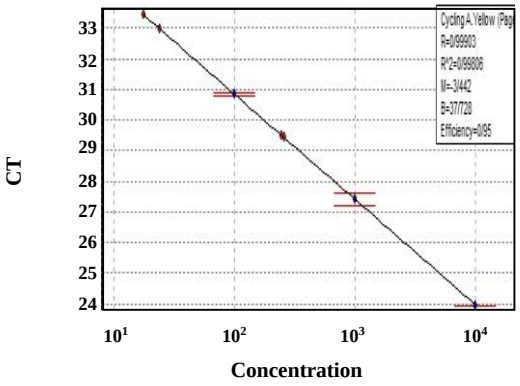

Fig. 2: Standard curve of qPCR amplification of serially diluted artificial inoculation showed as log of cells/g.

98 % having R-square value as 0.987. Another calibration curve was constructed for evaluating the effectiveness of the real-time PCR method using the artificially contaminated chickens; the slope of the curve was -3.442 and the amplification efficiency was 95 % having R-square value as 0.99 (Fig 2). The CT values obtained using genomic DNA were higher, which may be because of the losses over purifying DNA. The R^2 values were between 0.96–0.99, which indicates that the linearity of TaqMan qPCR assays. The assay targets the *prfA*gene, which has a detection limit of 18 cell/reaction; the inoculation sample assay revealed 2.7×10^1 CFU/g.

Comparison of qPCR and standard culture methods for detecting L. monocytogenes in naturally contaminated samples

In total, 100 naturally contaminated chicken rinses were analyzed by the standard culture and qPCR methods (before and after the enrichment step). Among the 100 specimens examined for *L. monocytogenes*, 13 (13 %) registered positive in the standard culture method, whereas 15 (15 %), and 17 (17 %) were positive in the qPCR method before and after enrichment, respectively. In addition, the specimens were examined using the conventional PCR method following enrichment for determination of the "true" positive specimens for comparative goals. Table 3 illustrates the results obtained using the qPCR, PCR, and standard culture methods (gold standard test) for the presence of *L. monocytogenes*.

Evaluation of qPCR

The following tables (Tables 4 and 5) are crosstabs of the culture test (T1), enrichment/real-time PCR test (T2), and direct/real-time PCR test (T4), which indicate the frequency and % of each test. Among the 100 tested samples, 87 (87 %) were negative as indicated by the culture method, 83 (95.4 %) samples were negative as indicated by T2, and 4 (4.6 %) samples changed to a positive result in T2. In addition, 13 (13 %) samples were positive as determined by the T1 test, whereas all samples remained positive as indicated by the T2 test. The Kappa test demonstrated that there was a significant relation between the T1 and T2 tests; the Kappa measure was 0.844 (ASE=0.076, 95%CI= (0.695, 0.992), p< 0.001). Moreover, Fisher's exact test showed that there was a relationship between the T1 and T2 tests at the level of 5 % ($p < 0.001$). The area under the curve (AUC) was obtained 0.98.

Based on the ROC curve (T1 and T2), it was observed that the AUC curve was 0.98 and the 95 % CI (confidence interval) for the curve was (0.995, 0.999). The values of the sensitivity and specificity were 0.99 and 0.954, respectively; the PPV and NPV were 0.765 and 1.000, respectively. In addition, the stratified bootstrap replications demonstrated a 95 % CI for sensitivity and specificity, which were (0.908, 0.989) and (1, 1), respectively.

According to Table 7, all positive samples obtained in T1 were also positive in T4. In addition, among 87 (87.0 %) of the negative samples from T1, only 85 (97.7 %)

Table 3: Detection of L. monocytogenes in chicken carcass samples (n=100) by cultural, PCR and qPCR methods.

Samples	No of samples	Positive samples (%)				
		Culture	PCR		qPCR	
			A*	B	C	D
Chicken carcass	100	13	15	17	15	17

**A and C: before enrichment, B and D: after enrichment*

Table 4: Crosstab of culture test (T1) and enrichment/real-time PCR (T2).

T1 \ T2		Negative	Positive	Total
Negative	N	83	4	
	Row (%)	95.4	4.60	87
	Column (%)	100	2.35	87.00
	Total (%)	83	4.00	
Positive	N	0	13	
	Row (%)	0	100	13
	Column (%)	0	76.50	13.00
	Total (%)	0	13.00	
Total		83 (83.00)	17 (17.00)	100

Table 5: Crosstab of culture test (T1) and direct/real-time PCR (T4).

T1 \ T2		Negative	Positive	Total
Negative	N	85	2	87 (87.0)
	Row %	97.7	2.3	
	Column %	100.0	13.3	
	Total %	85.0	2.0	
Positive	N	0	13	13 (13.0)
	Row %	0.0	100	
	Column %	0.0	86.7	
	Total %	0.0	13.0	
Total		85 (85.0)	15 (15.0)	100

remained negative. The Kappa measure was 0.917 and was significant (ASE=0.058, 95%CI= (0.804, 1) $p < 0.001$). Furthermore, Fisher's exact test indicated a significant relation between T1 and T4 ($p < 0.001$). The ROC curve of T1 and T4 was plotted, wherein the AUC was 0.99.

Predicted value of a positive test (%) = 100 * TP / (TP + FP)

Predicted value of a negative test (%) = 100 * TN / (TN + FN)

Discussion

In this study, 100 chicken carcasses were examined in terms of the existence of *L. monocytogenes* with the standard method, a rapid method combining culture and real-time PCR (with enrichment), and direct/real-time PCR (without enrichment). The *L. monocytogenes* were detected in our assay as in the previous studies [1, 16, 22, 25].

The assay targets the *prfA* gene, which has a detection limit of 18 cell equivalents per PCR reaction, and

is capable of detecting 27 CFU/25 g in a chicken in less than 48 hours (Fig. 2).

In some studies, the limit of detection was between 1–10 cell/reaction, which corresponds to 10^2 cells/g, 158 CFU/g,1–5 CFU/25 g, 5 copies per PCR, 10^0–10^2 CFU/g [1, 16, 22, 26, 29, 37].

All samples were evaluated using three methods including standard culture, PCR and qPCR. In their review by PCR and qPCR, after enrichment stage, 2 and 4 positive cases respectively were isolated. However, standard culture failed to isolate *Listeria monocytogenes* from these specimens. In addition, all cases reported positive in standard culture were also positive in PCR and PCR testing. In the examination of samples by PCR and qPCR without enrichment, positive cases were reported.

Among the 100 chicken rinses tested, 13 (13 %) specimens were positive in terms of *L. monocytogenes* and 87 (87 %) were negative as determined by the culture method; 17 (17 %) samples were positive in terms of *L. monocytogenes* as determined by the enrichment/real-time PCR, and 15 (15 %) specimens were positive in terms of *L. monocytogenes* as determined by the direct/real-time PCR (without enrichment) method.

Four false negative results were obtained in the enrichment/real-time PCR method and two false negative results in the direct/real-time PCR. One of the possible reasons for this outcome is that the enriched chicken/sample allows the living cells for growing and avoiding a false negative because of the trace amount in the primary specimen; hence, a condition of cell injury often failed to identify the pathogen [38]. Therefore, the enrichment is required to rise the sensitivity of the qPCR approaches, in case the levels of *L. monocytogenes* are low in the sample. Therefore, one of the disadvantages of detecting PCR is its failure to differentiate between alive and dead cells [39].

We set the standard curve and artificially inoculated the chickens, which represented a linear relation between the CT values and the logarithm corresponding to the level of objective DNAs or cells. The detection limit determined for *L. monocytogenes* was 27 CFU/g. Therefore, the enrichment/real-time PCR technique is presented as the best alternative for the standard method of testing as it also allows the estimation of the quantity of living bacteria. Moreover, chicken examined directly using the qPCR technique can be at the same time incubated in such a way that the samples that test negative can be tested further by qPCR after the enrichment step [1, 15, 26, 27, 32, 37, 40].

CONCLUSIONS

Generally, the outcomes achieved in current research for the presence of *L. monocytogenes* using 100 naturally contaminated chicken rinses focus on the fact that qPCR with enrichment is a more sensitive and accurate test as compared to the standard culture and conventional PCR methods. From the similarity of the results of PCR and qPCR, without the enrichment stage, it can be concluded that the DNA of the bacteria killed in the specimens was due to the presence of previous contamination, which can be determined by determining the number of copies of DNA by qPCR. The severity of contamination indicates the health status of the foodstuff. Over all, it can be said that the sensitivity of time and accuracy in diagnosis can be effective in choosing the type of test.

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