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Rapid Detection of *Listeria monocytogenes* in Ground Turkey Meats by Immunomagnetic Separation (IMS) and Real Time- Polymerase Chain Reaction (RT-PCR)

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Abstract : An immunomagnetic separation (IMS) and real-time polymerase chain reaction (RT-PCR) method was used to detect *Listeria monocytogenes* (*Lm*) in ground turkey meats. The IMS beads coated with monoclonal anti-*Lm* antibodies were used for bacterial separation. The primers and probes targeting the hemolysin O (*hyl A*) gene were used in the RT-PCR. The following five sets of samples containing *Lm* at dilutions of (10^0,10^1, 10^2, 10^3, 10^4 CFU) were tested by RT-PCR: 1) bacterial suspension without food sample and not subjected to IMS, 2) bacterial suspension without food sample but subjected to IMS, 3) bacterial sample with food sample but not subjected to IMS, and 4) bacterial suspension with food sample and subjected to IMS. In the RT-PCR, all samples that were spiked with 10 CFU and higher, showed positive signals. A drop in RT-PCR sensitivity varying from 5.0% to 9.3% was observed when the bacterial suspension was subjected to IMS. With respect to food samples spiked with *Lm*, the drop in sensitivity ranged from 11.1% to 14.0% as compared to samples containing the bacterial suspension alone. A total of 60 ground turkey meat samples were then studied without culture enrichment. This study suggests that combining IMS with RT-PCR significantly reduced the detection time of *Lm* to approximately 4 hours in ground turkey meats.

Key words: *Listeria monocytogenes, hemolysin, immunomagnetic separation; real-time PCR; turkey ground meat.*

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Introduction

Approximately 76 million human cases of food-borne illness are estimated to occur in the U.S.A. each year, and of these about 2,500 are caused by *Listeria monocytogenes (Lm).* Nevertheless, *Listeria* is responsible for approximately 27.6% of the total deaths attributed to food-borne illness (Doyle 2001)**.** Pregnant women, newborn babies, elderly people and people with weak or suppressed immune systems, AIDS, diabetes and kidney disease, are especially at high risk for contracting listeriosis. Indeed immuno-compromized patients can be up to 500-1000 times more susceptible to listeriosis than the general population (Jensen et al**.** 1994**;** Hof 2003)**.** In the year 2000, Center for Disease Control and Prevention (CDC&P) reported that of all food-borne pathogens as tracked by CDC&P, *Lm* had the second highest case fatality rate of 21% and the highest hospitalization rate of 90.5%, (USDA report 2003)**.** Such a high mortality rate makes *Lm* a notable public health hazard of primary concern.

The ubiquitous distribution of this pathogen in the nature, and its ability to survive refrigeration (4°C) temperatures, and its tolerance to certain food preservatives, makes elimination of *Lm* from foods very difficult (Bansal 1996**).** In early 1980s, several large outbreaks have occurred which prompted federal regulatory agencies to issue a "Zero Tolerance" policy for *Lm*. The critical issues facing the implementation of any "zero-tolerance" policy relates to the lack of reliable and rapid procedures for the detection of low numbers of *Listeria* in foods (Leonard et al. 2003). Conventional culture-methods are highly labor-intensive and time-consuming requiring 5 to 10 days to complete (Klein and Juneja 1997)**.** Hence the development of rapid and sensitive detection method is crucial. These methods should also be amenable to high number of samples and must be cost effective. The

immunomagnetic separation (IMS), represents a feasible method for rapid isolation and concentration of *Listeria* cells. The RT-PCR is highly specific and is capable of detecting as low as 1 copy of the target nucleic acid in relatively short time compared to the culture-based detection methods (Rodriguez-Lazaro et al**.** 2004). However, RT-PCR detection of the bacteria in food samples is currently done following the pre-enrichment culture because of the inhibitors in food samples. The objective of this study was to develop a rapid, specific and sensitive method for detection of *Lm* in the turkey ground meats, eliminating the need for culture-dependent approaches. Such a method could represent a fast and valuable tool to the food industry and the regulatory bodies to monitor the presence of *Lm* in food and environmental samples which will allow not only rapid trace back of the source of contamination but also the rapid recall of the contaminated product.

Materials and Methods

Bacterial strains, culture media and growth conditions: The bacterial species used in this study was *Listeria monocytogenes* (ATCC 19111). The culture was suspended in brain heart infusion (BHI) broth containing 20% glycerol and stored at -80ºC. From this culture a small sample was inoculated on Columbia blood agar containing 5% (v/v) horse blood overlay, and incubated at 37°C for overnight. Next day, pure colonies were used to prepare live *Lm* suspension adjusted to McFarland's nephelometer tube No. 2 (approximately 600 million bacteria/ml). Ten-fold serial dilutions were prepared to obtain a set of five dilutions containing 1 x 10^4, 1 x 10^3, 1 x 10^2, 1 x $10^1$and 1 x 10^0 CFU mL^{-1}. A standard plate count was always performed to validate the accuracy of the dilutions.

Paramagnetic beads coated with monoclonal antibody against *Lm* serotypes (DYNAL Inc, Brown Deer, WI) were used. It was suspended in phosphate-buffered saline (PBS), at pH 7.4 with 0.1 % bovine serum albumin (BSA) and 0.02% sodium azide (NaN_3) before using.

A region in *Lm* hemolysin O (hly A) gene was used as a target for RT- PCR amplification (Rodriguez-Lazaro et al. 2003)**.** The forward primer (5′-CAT GGC ACC ACC AGC ATC T-3′), the reverse primer (5′-ATC CGC GTG TTT CTT TTC GA-3′) and the probe (5′-FAM –CGC CTG CAA GTC CTA AGA CGC CA-TAMRA-3′), were prepared by Integrated DNA Technologies, Inc (IDT Inc, Coralville, IA). The amplified product was a 64-base pair fragment, from 113 to 177 base pair (GenBank Accession Number M24199; Rodriguez-Lazaro et al. 2003).

Food sample preparation: Turkey ground meats were purchased from local grocery stores and weighed into portions of 25 g. Each portion was put into a Whirl Pack Stomacher Bag (Nasco, Fort Atkinson, WI) incorporating a filter barrier film. The USDA standard method for isolation of *Lm* was used to screen the food samples to eliminate naturally contaminated samples from the experiments. Each sample was mixed with 1 ml of appropriately diluted bacteria.

Filtration and centrifugation: A 225 ml of 1% buffered peptone water (1% BPW, Oxoid Inc, Ogdesburg, NY) was added to the mixed samples and blended in stomacher bags for 2 min at the medium speed. Using a filter, the suspension was divided into 5-50 ml centrifuge tubes and centrifuged for 2 min at 1500 x g to remove the food particulates. The supernatants were decanted into another sterile 50 ml tube and centrifuged again at 1500 x g for 20 min. The supernatants were discarded and the pellets of each tube were collected in a 1.5 ml microcentrifuge tube and resuspended by adding 1 ml PBS containing 0.05% Tween 20 (PBS-T).

Immunomagnetic separation: 20 µl of IMS suspension were added to each tube. The microcentrifuge tubes were then loaded into the Dynal Magnetic Particle Concentrator (Dynal MPC) without magnetic plate, and incubated at room temperature for 20 min with continuous mixing (approximately 38 cycle's min^{-1}). The magnetic plate was then inserted into the Dynal MPC to concentrate the beads on to the side of the tube (3 min were allowed for proper recovery). The supernatant was carefully aspirated and plated on Mox agar plate to test for uncaptured bacterial cells. After removal of the magnetic plate from the Dynal MPC, 1 ml of washing buffer PBS-T was added to each tube and incubated at room temperature with continuous mixing, this step was repeated twice.

DNA Extraction: In the final step of the IMS procedure, the immunocaptured beads were resuspended in 180 µl of the lysis buffer (20 mM Tris pH 8.0, 2.0 mM Na EDTA 1.2% Triton X-100) and heated at 95°C for 10 min and left to cool at 37°C. Then a suspension of 20 mg/ml lysozyme was added and incubated for 30 min at 37°C. The purification of the DNA was done by using QIAGEN genomic DNA purification kit (QIAGEN Inc. 27220 Turnberry Lane, Valenica, CA). The concentration of all DNA was measured by using a spectrophotometer (Beckman Coulter DU 7400, Fullerton, CA) at 260/280 nm ratio and the DNA molecular size was measured by using 2% agarose gel electrophoresis stained with 0.5µg/mL-ethidium bromide.

Real-Time Polymerase Chain Reaction: A 25µl reaction volume containing OmniMix hot start containing 1.5 U TakaRa hot start Taqpolymerase, 200 µM dNTP, 4 mM Mg Cl_2 and 25 mM hepes buffer pH 8.0, 0.25 µl of 50 nM primers, 1.25 µl of 100 nM probe, 5 µl of the target DNA and 18.25 µl of nuclease free

dH_2O. Reactions were run on Smart Cycler II (Cepheid Inc, Sunnyvale, CA) with the following thermal program: 2 min at 50℃, 10 min at 95℃, 50 cycles of 15 second at 95℃ and 1 min at 60℃. Cycle threshold (C_T) of bacterial suspension without IMS and food sample was used as a comparative reference for other samples.

Results & Discussion

, In this study IMS was evaluated as a substitute for culture enrichment and as a pre RT-PCR step. Table 1 shows the C_T values for different sample sets and their dilutions. Calculated changes in the C_T values of sets 2, 3 and 4 and the percentage drop in RT-PCR sensitivity in relation to set 1 is shown in Table 2. As mentioned in the materials and methods no food sample was included in the bacterial suspension of set 1 and RT-PCR was done directly without IMS. The resultant C_T values indicated positive signals at all dilutions. In a regression curve, the C_T values of this set indicated a strong linear correlation, over 4 log unit dynamic range ($R^2 = 0.988$). As compared to dilution 1, an increase of 3.5 C_T suggests a decrease of 1 log CFU (Table 1).

The bacterial capture efficiency of IMS in the absence of food was determined in set 2. Positive signals were detected at all dilutions except dilution 1 (1 CFU) (C_T = 0.00). An increase in the number of C_T from 1.3 at dilution 5 ($1x10^4$ CFU) to 3.30 at dilution 2 ($1x10^1$ CFU) was observed (see Table 2).

Table 1. Variations of C_T between different sets of the study.

D# No. Of CFU/MI	Set 1 W/O FOOD &IMS		Set 2 W/ IMS W/O FOO		Set3 W/FOOD &IMS		Set4 W/FOOD WO/IMS	
	C_T	SD	C_T		C_T	SI	C_T	SD
D1 1X 10^0	46.12	0.15	0.00		0.00	. (	0.00	
00D2 1X 10^1	35.55	0.91	38.85		40.51	(	0.00	0.00
D3 1X 10^2	31.71	0.14	34.66		35.53	(	0.00	0.00
D4 1X 10^3	28.34	0.13	30.03		31.73	(	0.00	0.00
D5 1 X 10^4	25.11	0.16	26.36		27.89	(	30.24*	15.12

D# = Dilution number (D1=1, D2=10, D3=100, D4=1000, D5=10,000 CFU)
SD = Standard Deviation.
Mean and SD of C_T based on three observations.
*2 out of 15 samples indicated positive signals.

Table 2. Increase of C_T values and % of dropped IMS sensitivity relative to set 1

D# No. Of CFU/ML	Set 1 Reference C_T		Set 2 IMS W/O Food		Set 3 IMS W Food		Set 4 Food W/O IMS	
	C_T		C_T		C_T		C_T	
D1	1	46.12	0.	00.00	0	00.00	(	00.0 (
D2	1	35.55	0.	03.30	0	04.96	1	00.00 (
D3	1	31.71	0.	02.95	0	03.82	1	00.00 (
D4	1	28.34	0.	01.69	0	03.39	1	NA
D5	1	25.11	0.	01.25	0	02.78	1	NA

SD = Standard Deviation.
NA = Not Applicable.

In set 3 samples containing spiked ground turkey meat was subjected to IMS and then analyzed by RT-PCR. Positive signals were observed with all samples except those spiked with dilution 1, (C_T = 00.00). In comparison between mean C_T of set 1 and this set, an increase of C_T value from 2.8 at dilution 5 to 4.9 at dilution 2 was observed (Table 2).

In set 4, the spiked food samples were analyzed directly by RT-PCR without IMS. With dilutions 1 to 3, no signals were observed, only 1 sample at dilution 4 ($1x10^3$ CFU) showed signal equivalent to C_T = 48.7, which is higher than that obtained from 1CFU of set 1, therefore it was considered as negative. Two samples out of the 15 tested in this set indicated positive signals of C_T (44.67 and 46.06 at dilution 5).

Discussion

In this study we have evaluated IMS separation procedure prior to RT-PCR as a rapid method for detection of *Lm* in ground turkey meats. Earlier reports using IMS separation techniques, followed by regular PCR method required over 24 hours to complete the identification (Hudson et al. 2001). The RT-PCR allows a highly specific and quantitative detection of target DNA. It is capable of detecting as low as 1 copy of the target microbe in relatively short time compared to the other culture-based detection methods (Rodriguez-Lazaro et al**.** 2004**)** as well as regular PCR procedure. However, these described procedures required some pre-enrichment. IMS beads have been used to eliminate the need for selective enrichment and the time required for conventional methods by less than 24 hours time (Mansfield 1993**)**. In this study, we have tested an assay method, based on IMS followed by real-time PCR for the rapid detection of *Lm* from ground turkey meats without the prior-enrichment step.

As indicated in the material and methods, neither food nor IMS was included in set 1 (containing bacterial suspension only). The C_T values were used as a comparative reference (control). The RT-PCR was able to detect positive signals from all bacterial dilutions used in this set. This finding agrees with that reported by Rodriguez-Lazaro et al. 2004). In the absence of food, filtration and centrifugation appeared to be adequate for the retrieval of a bacterial concentration detectable by RT-PCR.

The results of set 2 unfolded a limitation in IMS when employed at concentrations as low as 1 CFU. Therefore, pre-IMS enrichment step was considered vital to amplify the target microbe to a concentration detectable by IMS. Such limitation was also reported by Blankenfeld-Enkvist et al. 2002. In another study conducted in our laboratory, enrichment of <2CFU bacterial suspension for 4 hours increased the sensitivity of IMS to a detectable level (Nadeem 2005). In the same set, IMS was able to recover the target *Lm* cells from all other dilutions but an increase in the number of C_T was observed. This suggested that IMS was not able to capture the absolute number or an equivalent number of *Lm* cells required to give C_T values identical to those obtained with set 1. The increase in C_T values was always less than 3.5 i.e. less than one log. Table 2 indicates a negative correlation between the bacterial cell density and change in the C_T, the higher the bacterial density, the lower the increase in the resultant C_T value. According to the bacterial densities used in this study, the observed increase in C_T values ranged from 4.9% at dilution 5 to 9.3% at dilution 2 (see Table 2 above).

In set 3, IMS was included in the retrieval of *Lm* from the spiked food samples. Signals were detected from all samples that were spiked with a concentration of 10 CFU/25 g and higher. This limit of detection without prior-enrichment is comparable to that achieved by Uyttendaele et al**.** 1999. In this set, the C_T values increased at a rate of more than that was observed with set 2. Samples spiked with dilution 2 and 3 gave an increase in C_T values equal to 4.96 (13.95%) and 3.82 (12.04%), respectively, hence a drop by more than one log in IMS recovery rate would be expected. The increase in the C_T values of the samples spiked with dilution 4 and 5 was less than 1 log.

This study indicated that, the sensitivity of IMS is not a function of the density of the target microbe only but also that of the food samples. Since the IMS beads are claimed by the manufacturer to react against all *Lm* serotypes but shows a reduced reaction to all other *Listeria* species and we have used RT-PCR primer pairs targeting a unique sequence in the hemolysin gene of *Lm* it was possible to detect *Lm* specifically and reliably against the back ground of other *Listeria* species occurring in the food samples.

In set 4, the extraction of *Lm* from the spiked food samples was done without IMS. Filtration and centrifugation were not efficient in the isolation and concentration of the target, but when IMS was included, it allowed the assay to show reliable signals from all samples spiked with 10 CFU/mL^{-1} and higher. Such findings are comparable to those of (Hudson et al., 2001). These data suggests that IMS did play a vital role in the concentration and isolation of *Lm* from food samples and could be used to replace the culture step.

The sensitivity limit was 10 CFU/25g but the fidelity of IMS when quantification is required would be questionable. This agrees with the findings of Blankenfeld-Enkvist et al. 2002.

In this study, we were able to develop a rapid method based on IMS and RT-PCR, to detect *Lm* in food in about 4 hours. The combination of faster detection techniques using IMS and RT-PCR would be of great value for the assessment of the risks of food-borne listeriosis, minimizing the unnecessary recalls and destruction of valuable food products and a better design of food safety control.

The procedures described, detected all of the samples that contained 170 to 1700 CFU, but CT values indicated a 10-fold under estimation of the actual number of CFU. The efficiency of the IMS appeared to be adversely affected by the food-associated inhibitors. The methods described involve the IMS followed by RT-PCR enabled the detection of *Lm* without prior enrichment in less than 4 hours.

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