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Process Optimization for the Detection of *Listeria monocytogenes* and Listeriolysin O from Spiked Chicken Meat by Polymerase Chain Reaction

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ABSTRACT

The study was undertaken to standardize PCR assay for detection of *L. monocytogenes* and Listeriolysin O from livestock foods and compare its efficacy with conventional cultural methods. A set of primer derived from *iap* gene and *hly A* gene were used for detection of *L. monocytogenes* and Listeriolysin O in the PCR assay. Electrophoresis analysis revealed the specific amplification products at 131 bp and 456 bp respectively for *iap* and *hlyA.* Among the three different template preparation methods viz. genomic DNA extraction, heat lysis and lysis buffer methods, heat lysis method was simple, rapid and reliable. The specificity of the standardized PCR assay was tested by subjecting 8 isolates including *L. monocytogenes* and seven other non-*Listeria monocytogenes* bacteria. Only *L. monocytogenes* isolates gave specific product of 131 bp for *iap* and 456 bp for *hlyA* genes respectively. The sensitivity of the PCR assay was evaluated by subjecting serial 10-fold dilution of pure culture of *L. monocytogenes* from $4.0X10^7$ cfu /ml to 4.0 cfu /ml to PCR assay. The minimum detection level was found to be 4 cfu/ml. Four different broths were evaluated to assess their PCR compatibility. Two non-selective broths (BHI and TSB) produced bands in all four treatments, but they were found as light. Among selective broths, LEB gave very bright bands to treatment-1 and 2 and bright bands to other treatments whereas PALCAM medium gave bright bands to treatment-1 and 2, and light bands to other two treatments. In spiking studies the minimum detection level for *L.monocytogenes* and Listeriolysin O was 4.0 cfu by PCR in LEB broth incubated for 24 hrs. LEB broth gave better results than PALCAM when incubated for 18 and 24hr.

Keywords : *Listeria monocytogenes, Listeriolysin O, Spiked studies in chicken, PCR*

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INTRODUCTION

Listeria monocytogenes is capable of surviving and multiplying under diverse environmental conditions such as low temperature, high salt concentration and moderately low pH. The major source of infection is due to consumption of contaminated vegetables, meat, dairy products and seafood products with *Listeria* species. The risk of infection from contaminated food increases after refrigeration. *Listeria monocytogenes* primarily affects pregnant women, neonates, the immunocompromised and the elderly people; showing major symptoms like septicaemia, meningoencephalitis and abortion (Niederhauser *et al.* 1992). The average mortality of *Listeria monocytogenes* (30%) far exceeds the other common food borne pathogens such as *Salmonella enteritidis* (with a mortality of 0.38%), *Campylobacter* species (0.02-0.1%) and *Vibrio* species (0.005-0.01%) in terms of disease severity (Liu 2006).

The traditional isolation and identification procedures are most widely used methods for detection of food borne pathogens. Even though, detection of *L. monocytogenes* from foods can be achieved most authentically by cultural methods, these are time-consuming and laborious procedures. A number of immunological and nucleic acid based methods have been developed for the rapid identification of *Listeria monocytogenes* from livestock products. Among these, the PCR technique is identified as an attractive alternative for detection of *L. monocytogenes,* since it is specific, highly sensitive and eliminates the need for enrichment culturing. *Listeria monocytogenes* has several important virulence markers. Among them, Listeriolysin O (LLO) is one of the important marker encoded by *hlyA* gene and is essential for disruption of phagocytic vacuole and release of bacteria into cytoplasm. Another important marker is p60, encoded by *iap* gene which plays a vital role in intestinal invasion. This gene is indispensable for species-specific identification of *Listeria monocytogenes*. The detection of single virulence associated genes is neither sufficient nor adequate to identify *L. monocytogenes*. So, it is necessary to target the both genes (*hlyA* and *iap*) individually through PCR assay (Nishibori *et al.* 1995; Ritu Aurora *et al.* 2007).

L. monocytogenes has been isolated easily from clinical samples due to higher number, but it is very difficult to isolate from foods due to low number. The U.S. Food and Drug

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administration, the Food Safety and Inspection Service (FSIS) and the USDA developed a mandate level of 'zero-tolerance' for *Listeriae* in ready-to-eat foods including cooked and smoked seafood (USDA 2002). Hence, there is need to isolate even single organism in the foods, PCR assay only justify it. Therefore, the present work was undertaken to assess the prevalence of *L. monocytogenes* in livestock products using PCR technique.

MATERIALS AND METHODS

The *Listeria monocytogenes* strain was obtained from Microbial Type Culture Collection (MTCC), Chandigarh. Other cultures maintained in the Department of Veterinary Public Health, College of Veterinary Science, Hyderabad were utilized in this study. The list of cultures used in this study were *Listeria monocytogene, Yersinia enterocolitica, E.coli* O157:H7, *Salmonella typhi, Salmonella virchow, Salmonella enteritidis, Klebsiella* and *Staphylococcus aureus.* The isolates required for the study were maintained in Brain Heart Infusion (BHI) broth by subculturing at regular intervals and tested periodically for their purity, morphological and biochemical characteristics. Before the studies conducted, the media used in this study like Brain Heart Infusion broth, Listeria Enrichment Broth (LEB), PALCAM agar, PALCAM broth, Motility test medium, Triple Sugar Iron agar, Urease test medium, Citrate utilization test medium, Tryptic soya brothwere made aseptic by autoclaving121°C at 15 psi for 15 min. unless otherwise specified. The sterility of the media was checked by incubating C for 24 hrs.

To determine the most suitable method for preparation of template in PCR, different methods of template preparation were tried in this study. Genomic DNA extraction and bacterial lysis procedures were done using pure cultures of *L. monocytogenes.*

Genomic DNA extraction: Genomic DNA extraction was carried out by using the protocol of Ozbey *et al.* (2008).

Bacterial lysis by heat application (Boiling and Snap chilling method): About 1.5 ml of Listeria Enrichment broth culture of *L. monocytogenes* was taken in to a micro centrifuge tube. The tube was then centrifuged at 6000 rpm for 10 min and supernatant was discarded. 50 μl of sterile distilled water was added to the tubes and boiled in a water bath at 100^{0}C for 10 min. and immediately transferred on to ice. Then centrifuged at 13,000 rpm for 5 min. Supernatant is collected and used as DNA template. For PCR assay, 5 μl of the bacterial lysate was taken as template.

Bacterial lysis using lysis buffer 1 and 2: To 0.3 ml of Listeria enrichment broth culture of *L. monocytogenes*, about 0.1 ml of lysis buffer-1 (7.5 mg/ml of lysozyme and 750 U/ml of mutanolysin) was added and incubated at 37^{0}C for 30 min. Then 0.1ml of lysis buffer-2 (5 mg/ml of Proteinase K and 50 mg/ml of Sodium lauryl Sarcosinate) was added. Later it was incubated at 37 ^{0}C for 15 min followed by the addition of 50 μl of 3M Sodium acetate and 1 ml of ice-cold absolute ethanol for DNA precipitation. DNA was collected by centrifugation and pellets were dried and resuspended in distilled water.

Polymerase chain reaction (PCR)

Oligonucleotide primers: The primers used for the *iap* gene and *hlyA* gene for the detection of *L.monocytogenes* and Listeriolysin O respectively are shown in Table 1.

Standardization of PCR protocol: PCR amplification of *iap* and *hlyA gene* fragments of this organism was set up to 25 μl reactions. The PCR protocol was initially standardized by optimizing the concentration of the components of the reaction mixture in the PCR assay and by varying the annealing temperatures and cycling conditions.

Table 1: Primers used in the present study

Primers	Target gene	Length	Primer sequence	Amplification product(bp)
iap-F	iap	20	5′ ACAAGCTGCA CCTGTTGCAG 3′	131
iap-R	Iap	20	5′ TGACAGCGTGTG TAGTAGCA 3′	131
hlyA- F	hlyA	24	5′ GCAGTTGCAAGCGC TTGGAGTGAA 3′	456
hlyA-R	hlyA	24	5′ GCAACGTATCCT CCAGAGTGATCG 3′	456

Reaction mixture: The master mix was made up to 25 μl using molecular grade water. In this study, the template preparation was done throughout the experiment by heat lysis (boiling and snap chilling) method. PCR assay was performed in Eppendorf gradient Thermal cycler with a heated lid. The cycling conditions used for two sets of primers are given in the Table 2. PCR products were stored at -20^{0}C until further use.

Initially, PCR master mix was prepared with the following ingredients- 2.5 μl of 10X PCR buffer containing 15mM $MgCl_2$, 2.0 μl of 25mM of $MgCl_2$, 1 μl of 10mM dNTP mix, 2 μl of each forward and reverse primer (4 pmol/ μl) and 0.9 U/ μl of Taq DNA polymerase which was made up to 20 μl using molecular grade water. Then, this master mix was distributed to the PCR tubes and finally 5 μl of bacterial lysate was added as template.

The bacterial isolates were inoculated in BHI broth, incubated overnight at 37^{0}C and subjected to heat lysis treatment and 5μl of each bacterial lysate was subjected to PCR for the two primers.

Agarose gel electrophoresis: Agarose gel (1.5%) was prepared by boiling agarose in an appropriate volume of 1X TAE buffer. After cooling for about 3 min, ethidium bromide (Biogene, USA) was added to the agarose solution to a final concentration of 0.5 μg/ml. The molten agarose was then poured into the tray and the comb was fitted into the slots of the tray. The tray was kept undisturbed until the gel got solidified. The comb was then taken out carefully and the tray containing the gel was then placed in a submarine horizontal electrophoresis unit filled with 1XTAE buffer up to a level of 1mm above the gel surface.

About 5 μl of each PCR product was mixed with 2 μl of bromo phenol blue loading dye (6X) and loaded into each well. Electrophoresis was performed at 5 V/cm and the mobility was monitored by the migration of the dye. After sufficient migration, the gels were observed under UV transilluminator to visualize the bands. The PCR product size was determined by comparing with a standard molecular weight marker and was photographed by the gel documentation system.

Evaluation of enrichment broths: The two different selective enrichment broths, Listeria enrichment broth (LEB) and PALCAM broth and two non-selective broths (BHI and TSB) were evaluated to find out their suitability for PCR assay. The broths were inoculated with a standard culture of *L. monocytogenes* and incubated (37^{o}C, 24hr). The cultures were processed as given below.

Treatment-1: About 2 ml culture was taken and centrifuged at 500 rpm for 30 sec. The supernatant was discarded and pelleted cells were washed in 1 ml of PBS, resuspended in 1 ml of cold, sterile distilled water. Denaturation was done by heating at 96^{0}C for 15 min. Then, centrifuged at 12,000 rpm for 10 min and the supernatant was used as template.

Treatment-2: About 1.5 ml of BHI broth culture of *Listeria monocytogenes* was taken and centrifuged at 13,000 rpm for 5 min. The recovered pellet was washed twice with 1 ml of distilled deionized water and resuspended in 100 μl of distilled deionized water. Cell lysate was obtained after heating 98^{0}C for 10 min.

Treatment -3: About 1 ml of TSY broth culture of *Listeria monocytogenes* was taken and centrifuged at 7000 rpm for 5 min. Then the cells were washed with 1 ml of sterile distilled water, resuspended in distilled water and heat lysed at 95^{0}C for 5 min. Cell debris was pelleted by centrifugation at 7000 rpm for 5 min and the supernatant was transferred to sterile, clean tube and used as template.

Treatment-4: The bacterial cells grown on the selective agar plates were swabbed, suspended in 100 μl of water and pelleted by centrifugation for 5 min at 13,000 rpm. The bacterial pellet was resuspended in 100 μl of 0.1 M NaOH and 0.25% sodium dodecyl sulfate and heated for 17 min at 90^{0}C. After cooling, it was used as template.

Artificial inoculation studies (Spiking studies): Samples (poultry meat) intended for spiking studies were initially screened for the presence of *Listeria monocytogenes* by inoculating 10 gm of the sample in 90 ml of LEB. Remaining portion of the sample was stored at -20^{0}C for spiking studies. Samples confirmed to be free from *Listeria monocytogenes* were used for spiking.

Homogenized chicken was taken in eight sachets (each sachet of 10 gm) and divided into two groups each containing four sachets. Four samples from each group were spiked separately with different concentrations of *Listeria monocytogenes* at the rate of 400 cfu, 40 cfu, 4.0 cfu and 0.4 cfu per 10 gm of meat. 90 ml of LEB and PALCAM broths were added to each meat portion and incubated at 37^{0}C for 18 h and 24 h.

The aliquots from each broth were collected from the enrichment broths after 18 hrs and 24 hrs. The aliquots collected were processed by heat lysis. PALCAM agar plates were streaked with a loopful of culture from each broth. After an incubation period of 24hrs at 37^{0}C, the plates were observed for green color colonies surrounded by a black zone on PALCAM agar plates.

Analysis of results of spiking studies: The results obtained in PCR and cultural methods in experimental inoculation studies were analyzed and the sensitivity and the specificity of these detection methods were calculated as per the formulae described below:

1. Sensitivity (the ability to detect positive samples) = a/a+c
2. Specificity (the ability to detect negative samples) = d/b+d

a = True positive c = False Negative
b = False Positive d = True Negative

Identification of Listeria monocytogenes by conventional methods: The identification of *Listeria monocytogenes* was done using the following procedure. All the isolates that were suggestive of *L. monocytogenes* on plating media were streaked on PALCAM agar plates. The colonies which are green and surrounded by a black zone were subjected to various biochemical identification tests for confirmation. Motility was tested by inoculating in Motility agar medium.

RESULTS AND DISCUSSION

Initial experiments were conducted to standardize PCR conditions for detection of *iap* gene of *L. monocytogenes* and gene of Listeriolysin O template involved the empirical variation of annealing temperature (59-66°C), concentration of primer (2-10pmol), Magnesium chloride (15-25mM), template volume (2-8μl) and the cycling conditions. Standardization of PCR assay was done for *iap* gene using 5μl of bacterial lysate as template in a reaction mixture consisting of 2.5 μl of 10X PCR buffer containing 15mM Magnesium chloride, 2μl of 25mM Magnesium chloride, 1 μl each of dNTP, 2 μl each of primer and 0.5 μl of Taq DNA polymerase and a final reaction volume was made up to 25μl with molecular grade water. Initial denaturation at 95°C for 2 min, followed by 35 cycles each of denaturation at 95°C for 15 sec, annealing at 60°C for 30 sec and extension at 72°C for 90 sec with a final extension period of 10 min at 72°C was found to be optimum for obtaining the desired PCR amplicon of 131 bp from *iap* gene of *L. monocytogenes.* Electrophoretic analysis of the PCR product revealed the specific amplification of a 131 bp for *iap* gene of *L. monocytogenes* and 456 bp for *hly* gene of Listeriolysin O fragment, without the presence of any spurious product (Fig. 1 & 2).

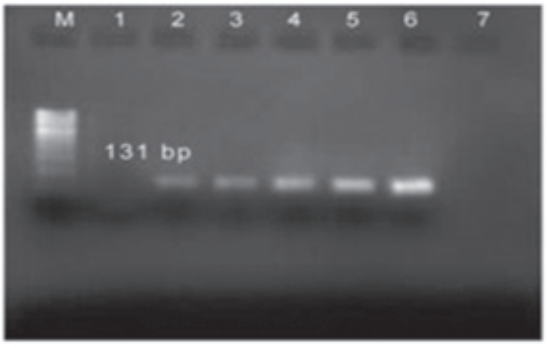

Fig.1: Standardization of PCR assay for detection of Listeria monocytogenes (iap)

Lane M : 100 bp DNA Ladder
Lane 2 : PCR product at an annealing temperature at 59°C
Lane 3 : PCR product at an annealing temperature at 60°C
Lane 4 : PCR product at an annealing temperature at 62°C
Lane 5 : PCR product at an annealing temperature at 64°C
Lane 6 : PCR product at an annealing temperature at 65°C

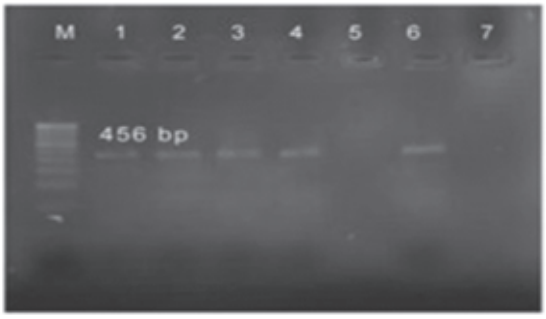

Fig.2: Standardization of PCR assay for detection of Listeriolysin O (hlyA)

Lane M : 100 bp DNA Ladder
Lane 1 : PCR product at an annealing temperature at 59°C
Lane 2 : PCR product at an annealing temperature at 60°C
Lane 3 : PCR product at an annealing temperature at 62°C
Lane 4 : PCR product at an annealing temperature at 64°C
Lane 6 : PCR product at an annealing temperature at 65°C

The primer sequences for *iap* and hlyA used by Rawool *et al.* (2007) and Ritu Aurora *et al.* (2007) was used for the standardization of PCR assay in this study that allowed amplification at 131 bp for *iap* and at 456 bp for *hlyA* genes respectively. These PCR products were stored at -20°C for further use.

Table 2: Cycling conditions used for two sets of primers

S.No	Step	*iap* (*L.monocytogenes*)	*hlyA* (Listeriolysin O)
1.	Initial denaturation	95°C / 2 min	95°C / 2 min
2.	Final denaturation	95°C/15 sec	95°C/15 sec
3.	Annealing	60°C/30 sec	60°C/30 sec
4.	Initial extension	72°C/1min. 30 sec	72°C/1min.30 sec
5.	Final extension	72°C/10 min	72°C/10 min
6.	Hold	4°C	4°C

Due to compository effect caused by complex composition of food, direct application of PCR assay results in poor sensitivity (Fluit *et al.* 1993) or no amplification products (Li *et al.* 1988). To simplify the procedure of template preparation and to improve efficiency of detection of pathogens, centrifugation and heat treatment, either individually or in combination were used for removal of PCR inhibitors from food matrices.

In this study, various efficient DNA template preparation methods were tried for detection of *L. monocytogenes* (*iap*) and Listeriolysin O(*hlyA*) in livestock products to exploit the potential of PCR assay. However, the routine use of PCR for detection of pathogens from food is modest because of various PCR-inhibitory components in food and media (Olsen 2000).

Three different DNA template preparation methods were evaluated to determine the most efficient one in identification of *L. monocytogenes* DNA from broth culture for PCR assay. Among the three methods, heat lysis method (boiling and snap chilling) gave better results than the genomic DNA extraction method. The other method i.e. using lysis buffer also gave acceptable results but the results are not comparable with heat lysis method. This is in accordance with the findings of Rossen *et al.* (1992), who reported that due to presence of lytic reagents like SDS and Triton X-100 above certain level reduced the detection limit ofpathogenic microorganisms and also had an inhibitory effect on PCR. Though, the genomic DNA extraction method is an ideal method, but it is time consuming and laborious and therefore not suitable for testing of more number of samples. In this study, we followed heat lysis method of template preparation, as it is simple, rapid and reliable for DNA template preparation.

The specificity of PCR assay was done for both *L. monocytogenes* and Listeriolysin O using specific primers targeting *iap* and *hlyA* genes by testing 8 isolates including *L. monocytogenes* and other 7 non-*L. monocytogenes* isolates. The specificity of the standardized PCR assay for primers *iap gene* was tested by subjecting seven other gram negative and gram positive organisms. *L. monocytogenes* yielded a specific PCR product of desired length (131 bp). No specific PCR product was obtained with any of the non-*L. monocytogenes* strains tested (Fig.3). Similarly, the specificity for primers *hlyA* genewas also tested by subjecting seven other gram negative and gram positive organisms. *Listeria monocytogenes* yielded a specific PCR product of desired length (456 bp). No specific PCR product was obtained from any of the non-*L. monocytogenes* strains tested (Fig. 4). Similar findings were reported by Ritu Aurora *et al.* (2007).

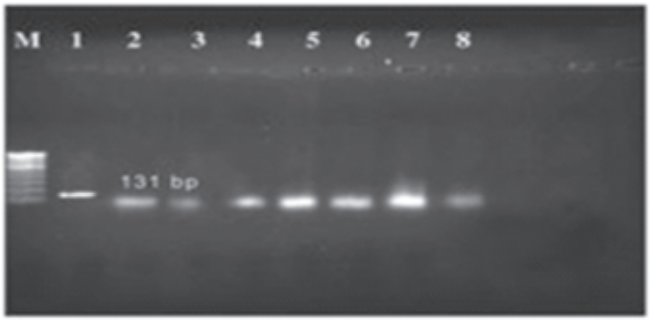

Fig.3: Specificity of PCR assay for detection of L.monocytogenes (iap):

Lane M : 100 bp DNA Ladder
Lane 1 : Listeria monocytogenes
Lane 2,3 : Yersinia enterocolitica, E.coli O157:H7
Lane 4,5 : Salmonella typhi, Salmonella virchow
Lane 6,7,8 : Salmonella enteritidis, Klebsiella, Staphylococcus aureus

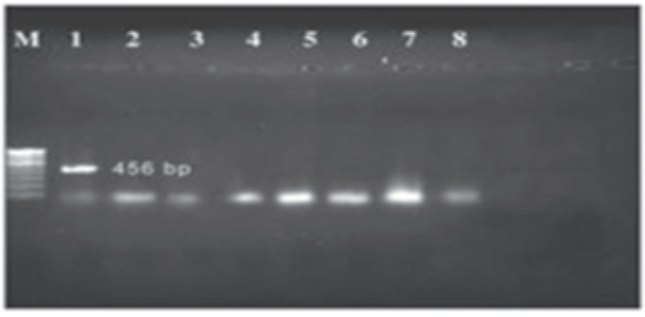

Fig.4: Specificity of PCR assay for detection of Listeriolysin O (hlyA)

Lane M : 100 bp DNA Ladder
Lane 1 : Listeria monocytogenes
Lane 2,3 : Yersinia enterocolitica, E.coli O157:H7
Lane 4,5 : Salmonella typhi, Salmonella virchow
Lane 6,7,8 : Salmonella enteritidis, Klebsiella, Staphylococcus aureus

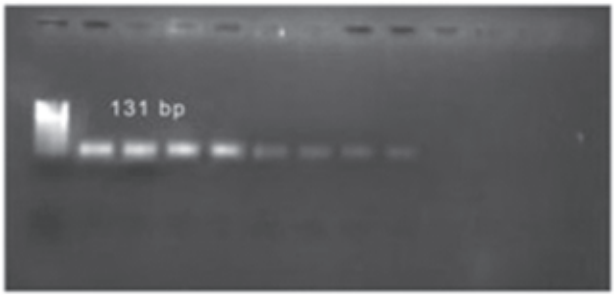

Fig.5: Evaluation of threshold sensitivity of PCR assay (iap)

Lane M : 100 bp DNA Ladder
Lane 1 : 4x107 cfu/ml
Lane 2 : 4x106 cfu/ml
Lane 3 : 4x105 cfu/ml
Lane 4 : 4x104 cfu/ml
Lane 5 : 4x103 cfu/ml
Lane 6 : 4x102 cfu/ml
Lane 7 : 40 cfu/ml
Lane 8 : 4 cfu/ml

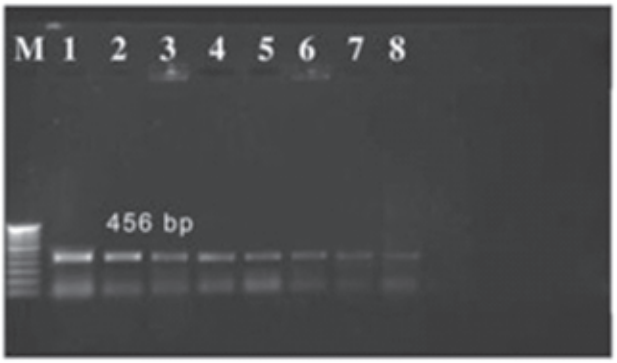

Fig.6: Evaluation of threshold sensitivity of PCR assay (hlyA)

Lane M : 100 bp DNA Ladder
Lane 1 : 4x107 cfu/ml
Lane 2 : 4x106 cfu/ml
Lane 3 : 4x105 cfu/ml
Lane 4 : 4x104 cfu/ml
Lane 5 : 4x103 cfu/ml
Lane 6 : 4x102 cfu/ml
Lane 7 : 40 cfu/ml
Lane 8 : 4 cfu/ml

The results of evaluation of threshold sensitivity of *L. monocytogenes* and Listeriolysin O by PCR assay are presented in Fig. 5 and 6. The sensitivity of the PCR was evaluated by subjecting serial 10 fold dilutions of a pure culture of *Listeria monocytogenes,* ranging from $4x10^7$ cfu/ml to 4 cfu/ml to PCR for two genes (*iap* and *hlyA).* The minimum detection level was found to be 4 cfu/ml. The intensity of band at the lowest detection level i.e. 4 cfu/ml was clearly discernible. Sensitivity and specificity are the critical parameters that define the accuracy of PCR assay (Hoorfar *et al.* 2004). The threshold sensitivity of PCR assay for detection of *L. monocytogenes* has been evaluated in pure culture and observed a minimum sensitivity level as 4 cfu/ml. Almost similar threshold sensitivity (5 cfu/g) was reported by Somen and Kashi (2003) in ice cream samples.

In PCR, false negative reactions occur due to presence of PCR-inhibitory components in foods and media and amplification of nucleic acid of dead organisms (Olsen 2000). To overcome this, single step enrichment method was used as a part of PCR assay. Even though, there are many reports on comparison between non-enrichment and enrichment broths for isolation of *L. monocytogenes* in traditional methods very limited reports are available on PCR compatibility of media. In this present study, the PCR compatibility of two non-enrichment broths (i.e. BHI and TSB) and two enrichment broths (i.e. LEB and PALCAM) were evaluated for detection of *L. monocytogenes* and Listeriolysin O. Four different treatments were tried for concentrating more target organisms in small test volume. The two non-selective enrichment broths i.e. BHI and TSB gave light bands for all four treatments. Among the two selective enrichment broths i.e. LEB and PALCAM, LEB gave very bright bands whereas, PALCAM gave light bands for treatments1 and 2. The LEB gave bright bands whereas, PALCAM gave light bands for treatments 3 and 4 (Table 3 and Fig. 7, 8, 9, 10). Balamurugan *et al.* (2006) reported that the MUVM (Modified University of Vermont Medium) and LEB broths gave bright bands while, PALCAM broth gave negative results in PCR due to presence of higher quantities of Ferric ammonium citrate, a potent inhibitor of PCR (Rossen *et al.* 1992). The superiority of MUVM and LEB over other media has also been reported by Brackett and Beuchat (1989); Bhilegaonkar (1998) and Chaudari (2001).

Table 3: Evaluation of enrichment broths for PCR compatibility

	Media tested			
Treatment	**BHI**	**TSB**	**LEB**	**PALCAM**
1	L	L	B+	B
2.	L	L	B+	B
3.	L	L	B	L
4.	L	L	B	L

L – Light; B – Bright; B+ - Very bright; Trt - 1 —>Heat lysis after two step centrifugation; Trt -2 —> Heat lysis after single step centrifugation; Trt – 3 —> First centrifuged, then heat lysed and centrifuged again; Trt – 4—> Heat lysis method using NaOH and Sodium dodecyl sulfate

Fig.7: Evaluation of PCR compatibility of Non-enrichment broths for the detection of iap

Lane M : 100 bp DNA Ladder
Lane 1 : BHI (Treatment-1)
Lane 2 : TSB (Treatment-1)
Lane 3 : BHI (Treatment-2)
Lane 4 : TSB (Treatment-2)
Lane 5 : BHI (Treatment-3)
Lane 6 : TSB (Treatment-3)
Lane 7 : BHI (Treatment-4)
Lane 8 : TSB (Treatment-4)

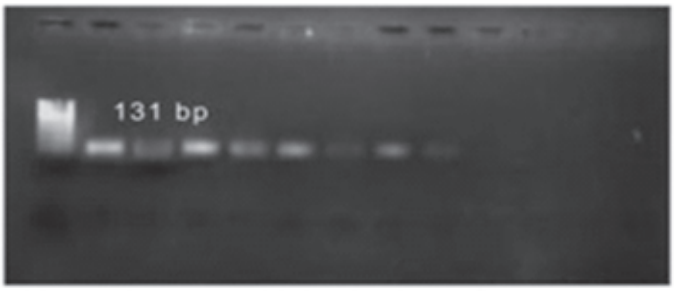

Fig. 9 Evaluation of PCR compatibility of enrichment broths for the detection of iap

Lane M : 100 bp DNA Ladder
Lane 1 : LEB (Treatment-1)
Lane 2 : PALCAM (Treatment-1)
Lane 3 : LEB (Treatment-2)
Lane 4 : PALCAM (Treatment-2)
Lane 5 : LEB (Treatment-3)
Lane 6 : PALCAM (Treatment-3)
Lane 7 : LEB (Treatment-4)
Lane 8 : PALCAM (Treatment-4)

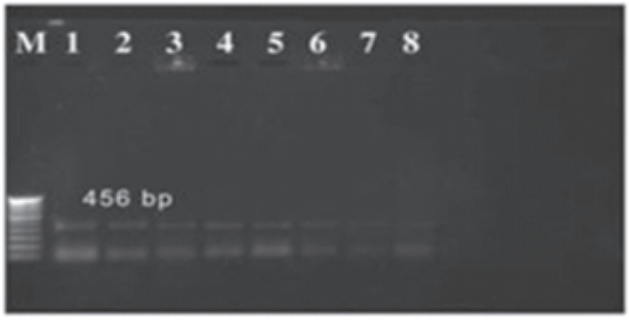

Fig. 8 Evaluation of PCR compatibility of Non-enrichment broths for the detection of hlyA

Lane M : 100 bp DNA Ladder
Lane 1 : BHI (Treatment-1)
Lane 2 : TSB (Treatment-1)
Lane 3 : BHI (Treatment-2)
Lane 4 : TSB (Treatment-2)
Lane 5 : BHI (Treatment-3)
Lane 6 : TSB (Treatment-3)
Lane 7 : BHI (Treatment-4)
Lane 8 : TSB (Treatment-4)

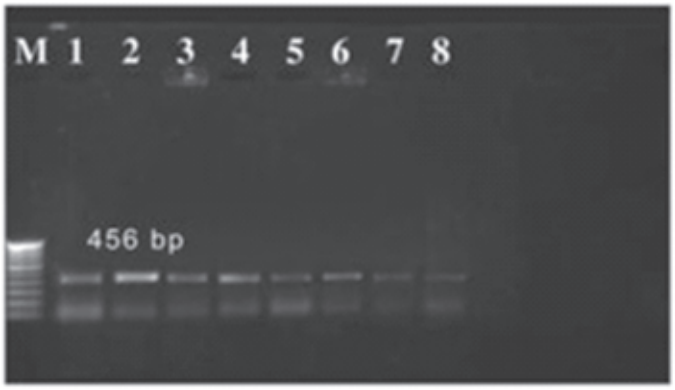

Fig.10 Evaluation of PCR compatibility of enrichment broths for the detection of hlyA

Lane M : 100 bp DNA Ladder
Lane 1 : PALCAM (Treatment-1)
Lane 2 : LEB (Treatment-1)
Lane 3 : PALCAM (Treatment-2)
Lane 4 : LEB (Treatment-2)
Lane 5 : PALCAM (Treatment-3)
Lane 6 : LEB (Treatment-3)
Lane 7 : PALCAM (Treatment-4)
Lane 8 : LEB (Treatment-4)

The homogenized chicken was inoculated with *L. monocytogenes* at the rate of 400 cfu, 40 cfu, 4.0 cfu, 0.4 cfu per 10 gm of chicken and transferred to two different enrichment media i.e. LEB and PALCAM broths. Both the broths were incubated at 37^{0}C for 18 hrs. and 24 hrs. Later they were subjected to PCR assay for *L. monocytogenes* and Listeriolysin O and conventional culture method for *L. monocytogenes* isolation and identification.

Table 4 : Organoleptic traits males and females of Indigenous chicken.

Selective broth	Time of Collected	Inoculation level (CFU / 10 gm of meat)							
		400		40		4.0		0.4	
		PCR	Cultural	PCR	Cultural	PCR	Cultural	PCR	Cultural
LEB	18 hrs	+	+	+	+	-	-	-	-
	24 hrs	+	+	+	+	+	-	-	-
PALCAM	18 hrs	+	+	-	-	-	-	-	-
	24 hrs	+	+	+	-	-	-	-	-

After 18 hrs enrichment, the positive results were obtained by PCR assay in LEB at a lowest inoculation level of 40 cfu and lower concentration than this did not show any amplification whereas, in PALCAM broth after 18 hrs of incubation PCR assay has given positive results up to lowest inoculation level of 400 cfu (Table 4). The cultural methods gave positive results only up to a lowest concentration of 40 cfu in LEB after 18 hrs incubation whereas, in PALCAM it gave positive result up to 400 cfu. After 24 hrs enrichment, the positive results were obtained at a lowest inoculation level of 4 cfu for LEB whereas, in PALCAM it gave positive results up to inoculation level of 40 cfu. The cultural methods gave positive results only up to a lowest concentration of 40 cfu after 24 hrs. of incubation whereas, in PALCAM it gave positive results up to 400 cfu.

During the spiking studies in the present study, homogenized chicken was inoculated at the rate of 400 cfu, 40 cfu, 4.0 cfu, 0.4

cfu per 10 gm of chicken and transferred to two different selective enrichment broths i.e. LEB and PALCAM. The PCR and cultural testing were carried after incubation for 18 and 24 hrs. The PCR results in spiking studies indicated that in LEB, *L. monocytogenes* and Listeriolysin O were detected at the inoculation level of 40 cfu after 18 hrs of incubation, whereas after 24 hrs incubation, the detection limit was 4 cfu. In PALCAM broth, *L. monocytogenes* and Listeriolysin O were detected at the inoculation level of 400 cfu after 18 hrs of incubation, whereas after 24 hrs incubation, the detection limit was 40 cfu. By cultural method positive results were obtained at 40 cfu and 400 cfu with LEB and PALCAM broths respectively both at 18 hrs and 24 hrs of incubation. These results indicated that LEB was found to be more suitable than PALCAM incubated for 24 hrs.

Higher sensitivity (2.5 cfu/gm) by PCR method was reported by Niederhauser *et al.* (1992). Slightly low sensitivity (10 cfu/gm) in spiking studies for detection of *L. monocytogenes* and Listeriolysin O by PCR reported by Wang *et al.* (1992) whereas Balamurugan *et al.* (2006) reported similar sensitivity (10 cfu) by PCR and cultural method. A sensitivity of 25 cfu/gm in ground beef samples by PCR assay after 24 hrs incubation was reported by Golsteyn Thomas *et al.* (1991). Several workers reported lower sensitivity by PCR method than the present study i.e. 10^3 cfu/gm in chicken samples by Navas *et al.*(2006) and 10^4 cfu/gm to 10^5 cfu/gm in chicken and ham samples by Moon *et al.* (2004). The sensitivity for detection of *L. monocytogenes* and Listeriolysin O in this study by PCR assay was high as incubation period increased. Golsteyn Thomas *et al.* (1991) also observed a 10-fold increase in sensitivity after 48 hrs incubation compared to 24 hrs.

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