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RESEARCH ARTICLE

Evaluation of various selective and non selective broths for detection of *Listeria monocytogenes* in pork and for PCR compatibility

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Abstract

The present study was undertaken to standardize PCR assay for detection of hly A (Listeriolysin O) and *iap*(p60) of *L.monocytogenes* from livestock foods and compare its efficacy with conventional cultural methods. A set of primer derived from *iap* gene and other set derived from *hlyA* 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* genes. Three different template preparation methods viz. genomic DNA extraction, heat lysis and lysis buffer methods were compared to determine the most sensitive, rapid and simple method suitable for PCR technique. The two non-selective broths (Brain Heart Infusion broth and Tryptone Soya Broth) and two selective broths (LEB and PALCAM) were also evaluated for PCR compatibility.

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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 septicemia, meningoencephalitis and abortion. 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 (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 because of the presence of the same in low numbers. The U.S. Food and Drug 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, 2003). 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 by using PCR technique, to evaluate the both enrichment and non-enrichment broths for PCR compatibility.

MATERIALS AND METHODS:

The *Listeria monocytogenes* strain was obtained from Microbial Type Culture Collection (MTCC), Chandigarh. 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 broth were made aseptic by autoclaving121^{o}C at 15 psi for 15 min. unless otherwise specified. The sterility of the media was checked by incubating at 37°C for 24 hrs.

To determine the most suitable method for preparation of template for use 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). *Listeria monocytogenes* culture grown on PALCAM agar plates was transferred into Eppendorf tube containing 300 µl sterile distilled water and it was incubated at 56^{0}c for 30 minutes. After the incubation 300 µl of TNES buffer and 200 µl/ml Proteinase K were added to this. The mixture was incubated at 56^{0}C for 30 minutes and added 500 µl of Phenol to the suspension. The suspension was shaked by hand for 5 minutes, and centrifuged at 11,600 rpm for 10 minutes. After centrifugation upper phase was carefully transferred into new Eppendorf tube. 0.1 volume of 3M Sodium acetate and 2.5 volume of absolute ethanol were added to this suspension and kept at -20^{0}c overnight. The suspension was again centrifuged at 11,600 rpm for 10 minutes and upper phase was discarded and the pellet was washed twice with 95% and 70% ethanol respectively and each step followed by 5 minutes centrifugation. The pellet was dried, re suspended in 50 µl of sterile distilled water and stored at -20^{0}c until further use.

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 minutes 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 minutes and immediately transferred on to ice. Then centrifuged at 13,000 rpm for 5 minutes. 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 (7.5mg/ml of lysozyme and 750U/ml of mutanolysin) was added and incubated at 37^{0}c for 30 minutes. Then 0.1ml of lysis buffer-2 (5mg/ml of Proteinase K and 50 mg/ml of Sodium lauryl Sarcosinate) was added. Later it was incubated at 37^{0}c for 15 minutes 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 p60 and Listeriolysin O of *L.monocytogenes* 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.

Reaction mixture:

The components of the reaction mix were finally optimized as given in the Table 8. 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 No 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 (4pmol/ 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 minutes, 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 at 37°c for 24hr. The cultures were processed as given below.

Treatment-1:

About 2 ml culture was taken and centrifuged at 500 rpm for 30 seconds. 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 minutes. Then, centrifuged at 12,000 rpm for 10 minutes 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 minutes. 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 minutes.

Treatment -3:

About 1 ml of TSY broth culture of *Listeria monocytogenes* was taken and centrifuged at 7000 rpm for 5 minutes. 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 minutes. Cell debris was pelleted by centrifugation at 7000 rpm for 5 minutes 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 minutes 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 minutes at 90^{0}c. After cooling, it was used as template.

SCREENING OF NATURAL SAMPLES

25 pork samples of each 20 gm and 25 pork swab samples were aseptically collected from local market and college farm.

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 **(Table 3).** Motility was tested by inoculating in Motility agar medium.

RESULTS AND DISCUSSION:

The primers targeting specific genes i.e. *iap* and *hlyA* used in PCR assay were standardized by optimizing the annealing temperatures, primer concentration, $MgCl_2$ concentration, template volume and cycling conditions. The electrophoretic analysis of the PCR product revealed the specific amplification of a 131 bp fragment, and 456 bp without the presence of any spurious product **(Fig. 1&2).** The primer sequences for *iap* and hlyA were used by Rawool et al (2007) and Ritu Aurora et al (2007) for the standardization of PCR assay which allowed amplification at 131 bp for *iap* and at 456 bp for *hlyA* genes respectively. These PCR products were stored at -20^0c for further use.

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 p60 (*iap*) and Listeriolysin O (*hlyA*) of *L.monoctyogenes* 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. 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.

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 p60 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 4 and Fig. 3, 4, 5, 6).** 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 . The superiority of MUVM and LEB over other media has also been reported by Chaudari (2001).

The U.S. Food and Drug Administration, the Food Safety and Inspection Service (FSIS) and the USDA developed a mandate to a level of ‘zero-tolerance’ for Listeriae in ready-to-eat foods including cooked and smoked seafood. Therefore, the detection method should have high level of sensitivity i.e. able to detect as low as one organism. Direct detection of *L.monocytogenes* in food samples by PCR presents a number of technical difficulties such as the levels of *L.monocytogenes* in foods might be low relative to the presence of large number of other bacteria due to non-uniformity distribution of *L.monocytogenes* in food products and involvement of nucleic acids from other bacteria and the food sample itself may inhibit PCR assays.

Use of selective enrichment media overcomes the difficulties inherent in direct PCR by increasing the amount of target DNA and diluting out non-Listeriae DNA and other PCR inhibitors which may present in samples (Olsen, 2000). It also ensures that DNA obtained from viable *L.monocytogenes* is detected. The enrichment step before the application of PCR increases the minimum detection level of organisms from food.

Even though, many sophisticated techniques like IMS, DNA hybridization and PCR-ELISA helped to increase the threshold sensitivity of PCR assay slightly, the inclusion of enrichment step enhanced the minimum detection level to much higher levels. A single selective enrichment step before PCR has been used in many studies (Balamurugan et al., 2006, Ritu Aurora et al., 2007 and Rawool et al., 2007). In the present study, enrichment broths like LEB and PALCAM broths were used to favour the growth of *L.monocytogenes* only.

Out of 25 samples each of pork and pork swab samples, PCR gave positive results for 4 (16%) and 2 (8%), whereas cultural method gave 2 (8%) and 1 (4%) positive results respectively (**Fig.7, 8, 9 &10**). Almost similar incidence (7.5%) was reported by Molla et al (2004) by cultural method whereas higher incidence i.e. 17.6% and

14% were reported by Autio et al (2004). The incidence of *L.monocytogenes* in the present study (16%) by PCR method was slightly higher than the incidence (14.3%) reported by Van Coillie et al (2004).

High incidence i.e. 41% and 47% has also been reported by and Hong et al (2007) respectively in pork samples by PCR method.

In this present study, two selective broths (LEB and PALCAM) were tried, of which LEB broth is able to detect very low levels of *L.monocytogenes*, which is encouraging. The present study also suggests the need for improving food safety through the implementation of hygienic measures at all levels from production to consumption with particular emphasis on ready-to-eat food items which require no further heat treatment.

Table. 1 Primers used in the present study (Ritu Arora *et al.,* 2007)

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

Table.2: Cycling conditions used for two sets of primers

S.No	Step	*iap* (*L.monocytogenes*)	*hlyA* (Listeriolysin O)
1.	Initial denaturation	95^0C / 2 min	95^0C / 2 min
2.	Final denaturation	95^0C/15 sec	95^0C/15 sec
3.	Annealing	60^0C/30 sec	60^0C/30 sec
4.	Initial extension	72^0C/1min. 30 sec	72^0C/1min.30 sec
5.	Final extension	72^0C/10 min	72^0C/10 min
6.	Hold	4^0C	4^0C

Table.3: Biochemical tests

S.No.	Test	Typical reaction of *Listeria monocytogenes*
1.	Indole	Positive
2.	Methyl Red	Positive
3.	Voges proskeur's	Positive
4.	Citrate utilization	Negative
5.	Lactose	Positive
6.	Saccharose	Positive
7.	Dextrose	Positive
8.	Gas production	Negative
9.	H_2S production	Negative
10	Nitrate reduction	Negative
11.	Urease	Negative
12.	Motility	Positive
13.	CAMP test	Positive
14.	Haemolysis	Positive (β type)

Table 4. Evaluation of enrichment broths for PCR compatibility

Treatment	**Media tested**			
	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

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

Fig.3: 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)

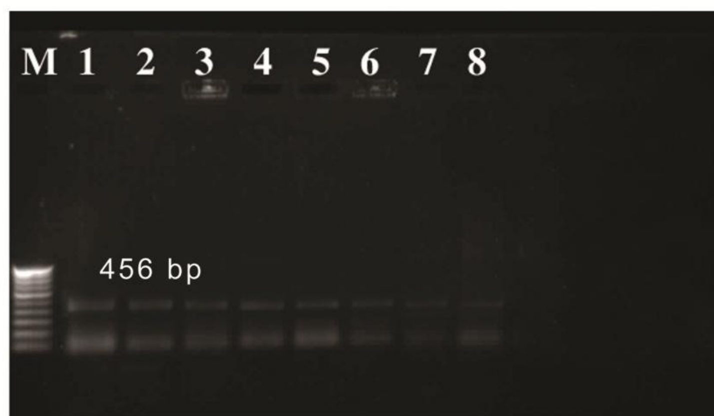

Fig.4: Evaluation of PCR compatibility of Non-enrichment broths for the detection of hlyA

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)

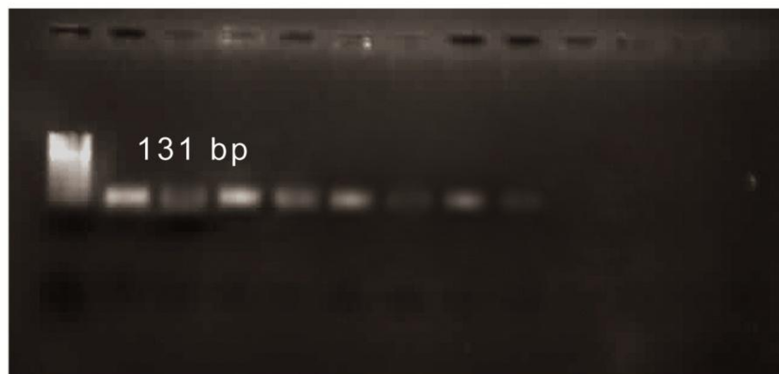

Fig. 5: Evaluation of PCR compatibility of enrichment broths for the detection of iap

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)

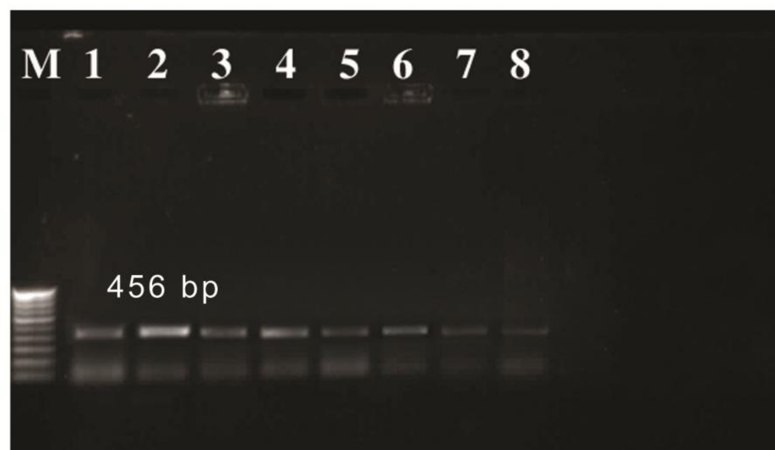

Fig. 6: Evaluation of PCR compatibility of enrichment broths for the detection of hlyA

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)

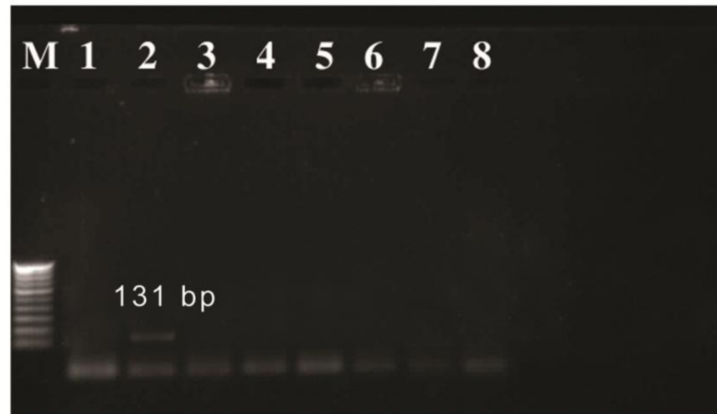

Fig.7: Results of pork samples for L.monocytogenes(iap):

Lane M : 100 bp DNA Ladder
Lane 2 : Pork samples showing positive results

Fig.8: Results of pork samples for Listeriolysin O (hlyA)

Lane M : 100 bp DNA Ladder
Lane 4 : Pork samples showing positive results

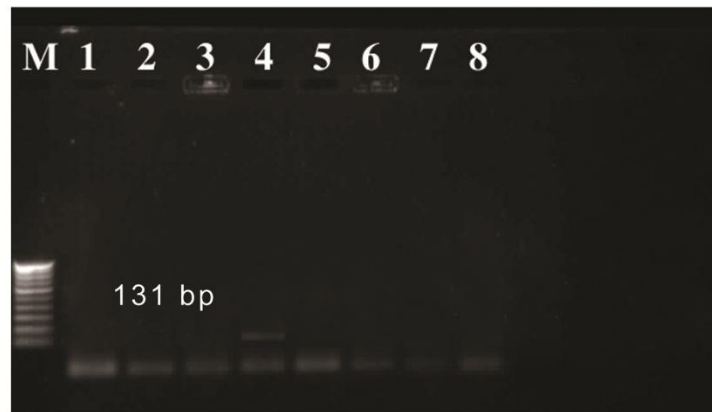

Fig.9: Results of pork swab samples for L.monocytogenes(iap):

Lane M : 100 bp DNA Ladder
Lane 4 : Pork swab samples showing positive results

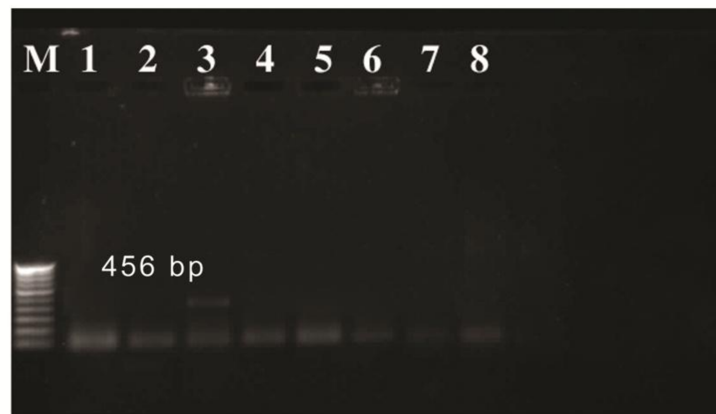

Fig.10: Results of pork swab samples for Listeriolysin O (hlyA)

Lane M : 100 bp DNA Ladder
Lane 3 : Pork swab samples showing positive results

In conclusion:

PCR was found to be a suitable test for screening of food samples for *L.monocytogenes* in a rapid way. Overall sensitivity of PCR was higher than cultural method and amenable to automation. 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. Among the two non-selective enrichment broths (Brain Heart Infusion broth and Tryptone Soya Broth) and two selective broths (LEB and PALCAM) were evaluated, BHI, TSB, PALCAM gave light bands and LEB gave very bright bands by PCR.

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