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Detection of *Listeria monocytogenes* from freshwater fish, prawn and chicken meat by direct nested PCR

SANJOY DAS[1,2]*, V.P. SINGH[1], KEDUZOL LTU[1], S. KATHIRESAN[1], BHASKAR SHARMA[1] and K.N. BHILEGAONKAR[1]

[1]Division of Bacteriology and Mycology, Indian Veterinary Research Institute, Izatnagar, - 243122, (UP), India
[2]MFB Division, Central Institute of Fisheries Technology, Matsyapuri P.O., Cochin- 682029, India (present address).

Abstract

Three different methods of processing samples from freshwater fish, prawn and chicken meat were compared with nested PCR for the detection of *Listeria monocytogenes*. Nested PCR failed to detect the organism from freshwater fish, prawn and chicken meat when processing was done by boiling lysis method, but it could detect the organisms from freshwater fish, prawn and chicken meat up to the spiking level of 10^4, 10^3 and 10^5 cfu·g^{-1}, respectively while processing of sample was done by phenol extraction without enrichment. However, using phenol extraction after enrichment, the sensitivity was found higher than without enrichment and it was possible to detect up to the spiking level of 10^2, 10 and 10^3 cfu·g^{-1} in case of fish, prawn and chicken meat, respectively.

Introduction

Listeria monocytogenes is an intracellular organism responsible for a number of infections viz. abortion, encephalomyelitis, arthritis, gastroenteritis, meningitis and conjunctivitis in animals and human beings (Vazquez-Boland et al. 2001). Apart from that, it is also important from the food safety point of view. Food products for export should be free from this pathogen, and the detection of *L. monocytogenes* from different food samples is of utmost importance.

Among six species of the genus *Listeria* (*L. monocytogenes*, *L. innocua*, *L. ivanovii*, *L. grayi*, *L. seeligeri* and *L. welshimeri*), the sole human pathogenic species is *L. monocytogenes*. In ruminants, the disease is very often evidenced by a specific nervous disorder called 'circling calf syndrome' (Vishwanathan and Ayyar, 1950). *L. monocytogenes* is very widely distributed in nature and its transmission to the human being is mostly through the ingestion of food items contaminated with this organism. The presence of the pathogen has been reported in various food products including fish, prawn and chicken meat causing outbreaks and sporadic infections (Nagi and Verma, 1967; Riedo et al. 1994; Ericsson et al. 1997; Dhanashree et al. 2003). Storage of food at low temperature cannot prevent the growth of the organism and it can grow even at refrigerated temperatures (Junttila et al. 1988). Many regulatory authorities including the United States Food and

*Corresponding author. E-mail address: sanjoy125@yahoo.co.in

Drug Administration have imposed a zero tolerance policy to this pathogen and thus food products for export should be free from *L. monocytogenes* (Hitchinis, 1998). The prompt detection of the pathogen from different food items is crucial.

The conventional method for detection of *L. monocytogenes* is very time intensive and tedious. Moreover, sometimes the *Listeria* organism is present in food in a viable but not culturable (VBNC) state. Different immunological tests like enzyme linked immunosorbant assay (ELISA), enzyme immunoassay (EIA), latex agglutination test (LAT) etc have been used for successful detection of *L. monocytogenes*, but these tests are also time-consuming and cannot differentiate *L. monocytogenes* from *L. innocua* (Mattingly et al. 1988; Gangar et al. 2000). To overcome these problems, PCR and real-time PCR have been used for rapid, prompt and specific detection of this pathogen (Herman et al. 1995, Castillo et al. 2005). Due to the complex nature of food matrices, the different PCR inhibitory substances present in food very often interfere with the sensitivity of PCR assays (Rossen et al. 1992). In the present study, three different methods of processing samples were compared for the detection of *L. monocytogenes* from freshwater fish, prawn and chicken meat.

Materials and Methods

Samples

A freshwater fish 'Rohu' (*Labeo rohita*) and chicken meat used in this study were procured from the local markets of Bareilly, India, while the giant fresh water prawn (*Macrobrachium rosenbergii*) was procured from INA market complex, Delhi.

Organism

L. monocytogenes MTCC 657 was obtained from microbial type culture collection, Institute of Microbial Technology (IMTECH), Chandigarh and maintained on tryptic soy agar (TSA) slants at 4 °C.

Preparation of bacterial stock and artificial spiking

A loop full of culture of *L. monocytogenes* (MTCC 657) was inoculated into 10 mL of tryptic soy broth (TSB) and incubated at 37 °C for 18 hr. The culture was centrifuged at 6,500 x g for 10 min at 4 °C and then the pellet was suspended in 10 mL of phosphate buffered saline (PBS). The bacterial population of the suspension was determined using serial dilutions method on TSA. Based on the concentration of *Listeria*, an appropriate amount of suspension was mixed in PBS to prepare a stock suspension, which contained 10^9 cfu of *L. monocytogenes*·mL^{-1}. Artificial spiking of fish, prawn and chicken meat was carried out by 10 folds dilution of above stock and 100 µL of inoculum was used in each case of artificial spiking.

Ten g of each sample from fish, prawn and chicken meat were spiked with 10^8, 10^7, 10^6, 10^5, 10^4, 10^3 and 10^2 cfu of *L. monocytogenes*, separately along with controls. For spiking, 100 µL of inoculum was spread on the sample and the samples were left at room temperature for 1 hr to get the inoculum absorbed. Before spiking, the samples were also screened for the presence of *Listeria* spp. naturally by conventional isolation procedure of organism by ISO 11290 Part 1 method as mentioned in Scotter et al. (2001).

Processing of samples for PCR

The processing of samples for PCR was carried out using three different methods.

(i) Without enrichment boiling lysis method:

Ten g of spiked samples along with the control were macerated in 90 mL of sterile normal saline solution in a stomacher for 1 min. The sample was then centrifuged first at low speed (500 x g for 10 min) to remove the large particles and then at 7,500 x g for 10 min at 4 °C. The pellet was washed with sterile normal saline solution and suspended in 250 µL of autoclaved triple distilled water. It was then placed in a boiling water bath for 10 min and immediately chilled at -20 °C for 2 hr. After centrifugation at low speed (100 x g for 2 min), 10 µL of the supernatant was used as PCR template.

(ii) Phenol extraction without enrichment:

The processing of each sample from fish, prawn and chicken meat was carried out without enrichment using phenol extraction as per the protocol of Liu et al. (2003). The sample processing up to obtaining the pellet was carried out as done previously. The pellet was washed with normal saline solution and suspended in 500 µL of lysis buffer (0.1 M Tris-HCl, pH 8.0, 2 % Triton X-100, 0.25 % sodium azide) containing 2 $mg \cdot mL^{-1}$ lysozyme, incubated at 37 °C for 30 min followed by addition of 10 µL of proteinase K (20 $mg \cdot mL^{-1}$). After thorough mixing, it was kept at 56 °C for 2 hr in a water bath. 550 µL of phenol: chloroform: isoamyl alcohol (25:24:1) mixture was added and mixed well by inversion, centrifuged at 7,000 x g for 5 min at 4 °C. Then upper aqueous phase was collected in a fresh microcentrifuge tube and was precipitated with 2 volumes of chilled ethanol (-20 °C) and then kept at -20 °C for 2 hr. Subsequently, it was centrifuged at 10,000 x g for 10 min at 4 °C. Then the ethanol was decanted and 70% chilled ethanol was added. It was centrifuged again at 7,000 x g for 5 min at 4 °C. The ethanol was decanted. The nucleic acid pellet was dried in a vacuum concentrator, dissolved in 50 µL of autoclaved triple distilled water and kept at 4 °C for further use.

(iii) Phenol extraction after enrichment:

The maceration process was done in 90 mL of UVM modified *Listeria* enrichment broth (BD Difco, USA) in place of normal saline solution and then, it was incubated at 30 °C for 18 hr.

Ten mL of the enriched sample was used for further processing as carried out previously (without enrichment by phenol extraction). Finally, the nucleic acid pellet was dissolved in 50 µL of autoclaved triple distilled water and 10 µL of this was used as PCR template.

PCR assay

PCR assay was carried out as a two-step nested PCR. In the 1st step, the primers LM1 (5′-CCTAAGACGCCAATCGAA-3′) and LM2 (5′- AAGCGCTTGCAACTGCTC-3') were used whereas, the 2nd step PCR was performed with the primer pair LL5 (5′-AACCTATCCAGGTGCTC-3′) and LL6 (5′-CTGTAAGCCATTTCGTC-3′). These primers are based on the *hly* gene of *L. monocytogenes*, which amplify a 702 bp product in 1st step and a 267 bp product in 2nd step from the internal region (Border et al. 1990; Herman et al. 1995).

In the 1st step PCR, PCR reactions were carried out in 25 µL mixture, which contained 2.5 µL of 10X PCR buffer (100 mM Tris-HCL, 500 mM of KCl and 0.8 % Nonidet P40), 10 µL of respective template, 200 µM dNTP mix, 10 pmole of each primer, 1.5 mM of $MgCl_2$ and 1 U of *Taq* DNA polymerase (Fermentas). The template DNA was denatured at 94 °C for 5 min as initial denaturation followed by 30 cycles of 94 °C for 30 sec (denaturation), 50 °C for 1 min (annealing) and 72 °C for 1 min (extension). After completion of 30 cycles, reaction mixture was held at 72 °C for 5 min for final extension (Border et al. 1990).

For the 2nd step PCR, 2 µL of product from 1st step was taken as template in the reaction mixture (25 µL), which consisted of template (2 µL), 2.5 µL of 10X PCR buffer (100 mM Tris-HCl, 500 mM of KCl and 0.8 % Nonidet P40), 200 µM dNTP mix, 10 pmole of each primer, 1.5 mM $MgCl_2$ and 1 U of *Taq* DNA polymerase (Fermentas). The reaction was performed using a programme of an initial denaturation at 94 °C for 5 min followed by 30 cycles of denaturation at 94 °C for 30 sec, primer annealing at 55 °C for 30 sec and extension at 72 °C for 30 sec. The final extension was carried out at 72 °C for 5 min.

PCR products were resolved by agarose gel electrophoresis on 1.5% agarose gel containing 0.3 $\mu g \cdot mL^{-1}$ ethidium bromide. The electrophoresis was carried out at 100 volts for 80 min using 0.5 X TBE (45 mM Tris, 45 mM boric acid and 2 mM EDTA, pH 8.0). Finally, the gel was visualized and photographed using UV gel documentation system (Alpha Innotech Corporation, USA).

Results

In the present study using the method without enrichment boiling lysis, none of the samples of fish, prawn and chicken meat yielded any amplified product at any level of spiking. In contrast, the processing of samples by phenol extraction yielded a 267 bp amplified product in the samples of fish, prawn and chicken meat, and the sensitivity was 10^4, 10^3 and 10^5 $cfu \cdot g^{-1}$ as shown in Figs. 1, 2 and 3, respectively. However, for the enriched samples before processing with phenol extraction, a 267 bp fragment was also found in fish with 10^2 $cfu \cdot g^{-1}$ of *L. monocytogenes*, while it was up to 10^3

cfu·g^{-1} in chicken meat (Figs. 4 and 6). In the case of prawn, the organism could be efficiently detected even at the level of 10 cfu·g^{-1} (Fig. 5).

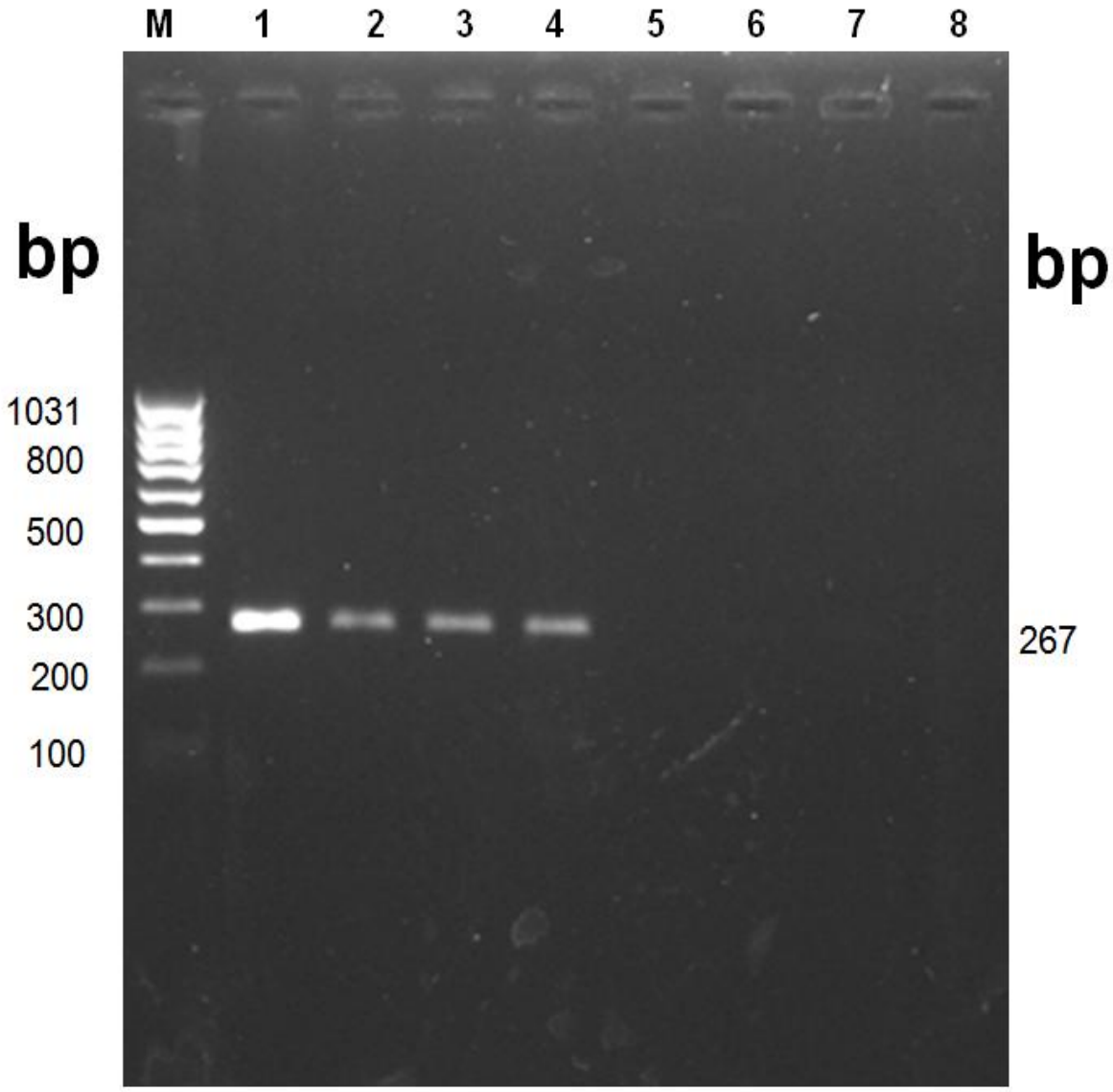

Fig. 1. PCR detection of *L. monocytogenes* in fish by phenol extraction without enrichment. Lane M:100 bp DNA ladder; Lane 1: Spiking level 10^7 cfu·g^{-1}; Lane 2: Spiking level 10^6 cfu·g^{-1}; Lane 3: Spiking level 10^5 cfu·g^{-1}; Lane 4: Spiking level 10^4 cfu·g^{-1}; Lane 5: Spiking level 10^3 cfu·g^{-1}; Lane 6: Spiking level 10^2 cfu·g^{-1}; Lane 7: Spiking level 10 cfu·g^{-1}; Lane 8: Control.

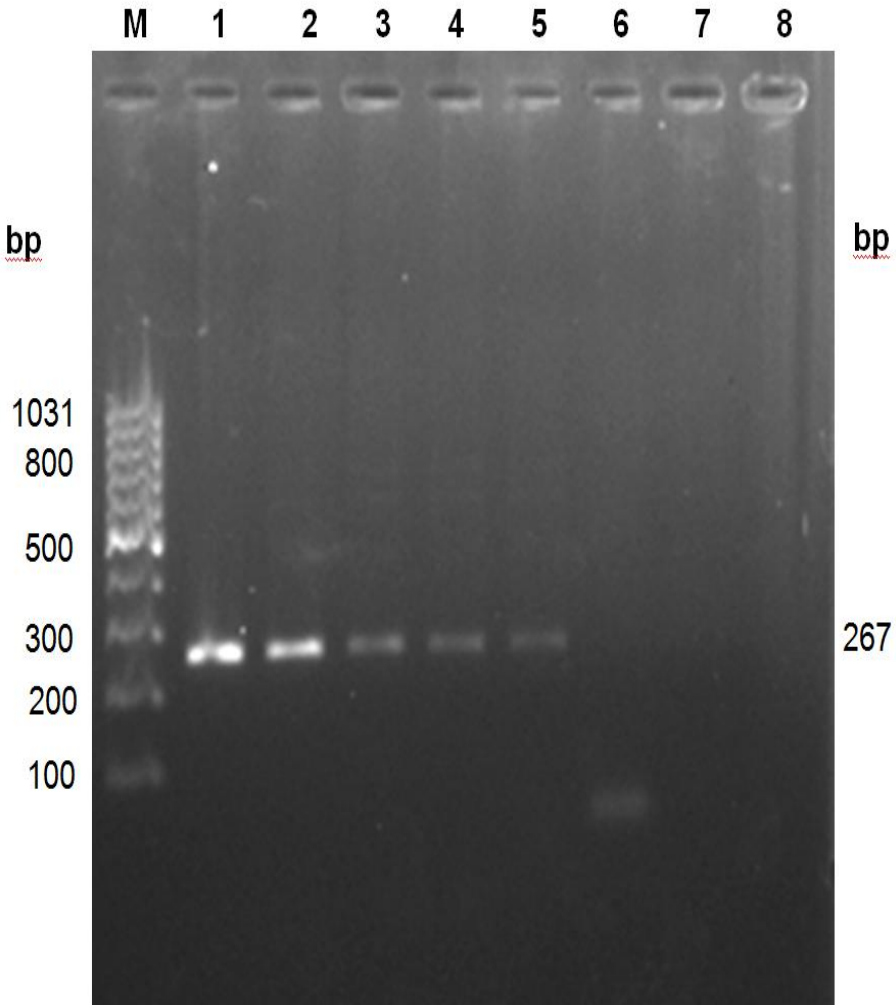

Fig. 2. PCR detection of *L. monocytogenes* in prawn by phenol extraction without enrichment. Lane M:100 bp DNA ladder; Lane 1: Spiking level 10^7 cfu·g^{-1}; Lane 2: Spiking level 10^6 cfu·g^{-1}; Lane 3: Spiking level 10^5 cfu·g^{-1}; Lane 4: Spiking level 10^4 cfu·g^{-1}; Lane 5: Spiking level 10^3 cfu·g^{-1}; Lane 6: Spiking level 10^2 cfu·g^{-1}; Lane 7: Spiking level 10 cfu·g^{-1}; Lane 8: Control.

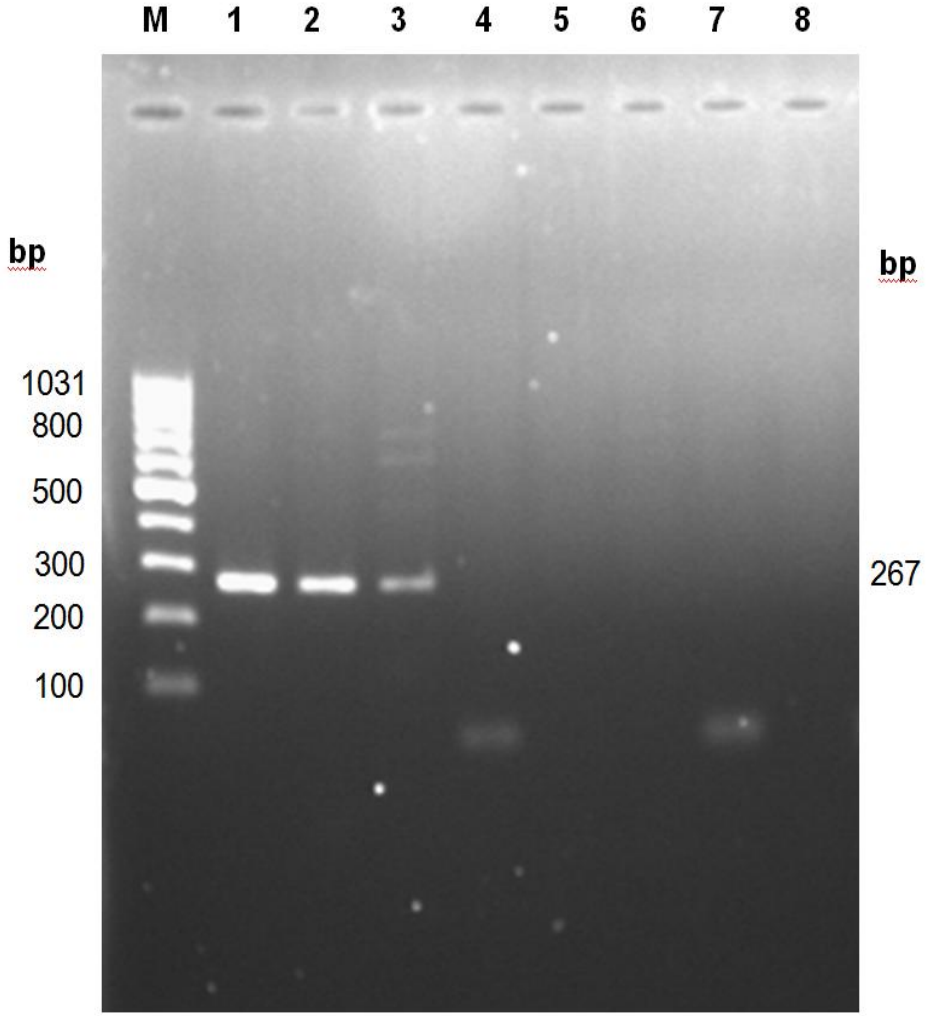

Fig. 3. PCR detection of *L. monocytogenes* in chicken meat by phenol extraction without enrichment. Lane M:100 bp DNA ladder; Lane 1: Spiking level 10^7 cfu·g^{-1}; Lane 2: Spiking level 10^6 cfu·g^{-1}; Lane 3: Spiking level 10^5 cfu·g^{-1}; Lane 4: Spiking level 10^4 cfu·g^{-1}; Lane 5: Spiking level 10^3 cfu·g^{-1}; Lane 6: Spiking level 10^2 cfu·g^{-1}; Lane 7: Spiking level 10 cfu·g^{-1}; Lane 8: Control.

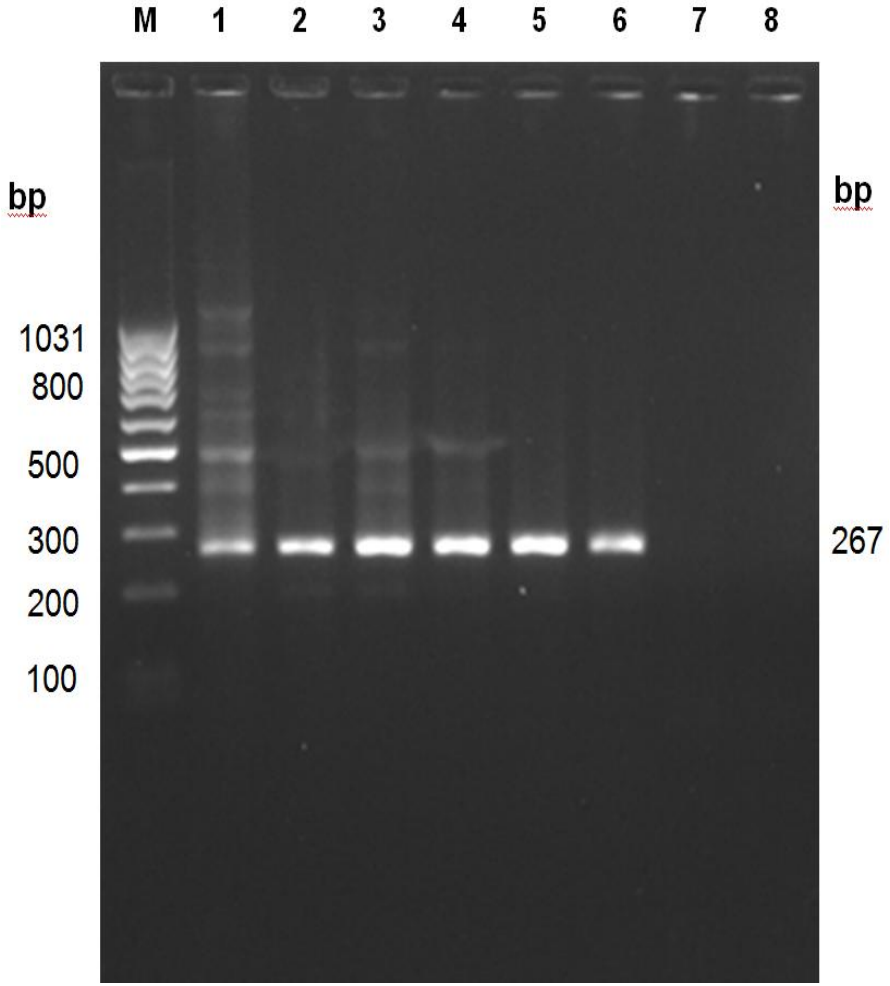

Fig. 4. PCR detection of *L. monocytogenes* in fish by phenol extraction after enrichment. Lane M:100 bp DNA ladder; Lane 1: Spiking level 10^7 cfu·g^{-1}; Lane 2: Spiking level 10^6 cfu·g^{-1}; Lane 3: Spiking level 10^5 cfu·g^{-1}; Lane 4: Spiking level 10^4 cfu·g^{-1}; Lane 5: Spiking level 10^3 cfu·g^{-1}; Lane 6: Spiking level 10^2 cfu·g^{-1}; Lane 7: Spiking level 10 cfu·g^{-1}; Lane 8: Control.

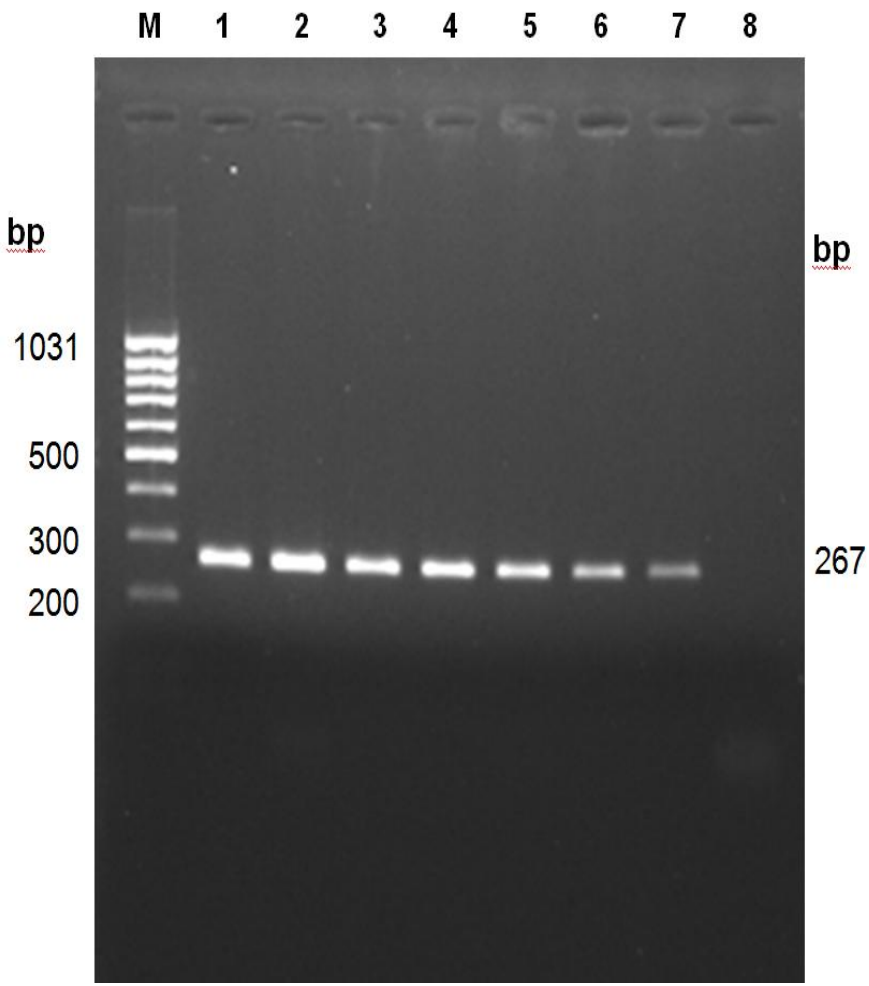

Fig. 5. PCR detection of *L. monocytogenes* in prawn by phenol extraction after enrichment. Lane M:100 bp DNA ladder; Lane 1: Spiking level 10^7 cfu·g^{-1}; Lane 2: Spiking level 10^6 cfu·g^{-1}; Lane 3: Spiking level 10^5 cfu·g^{-1}; Lane 4: Spiking level 10^4 cfu·g^{-1}; Lane 5: Spiking level 10^3 cfu·g^{-1}; Lane 6: Spiking level 10^2 cfu·g^{-1}; Lane 7: Spiking level 10 cfu·g^{-1}; Lane 8: Control.

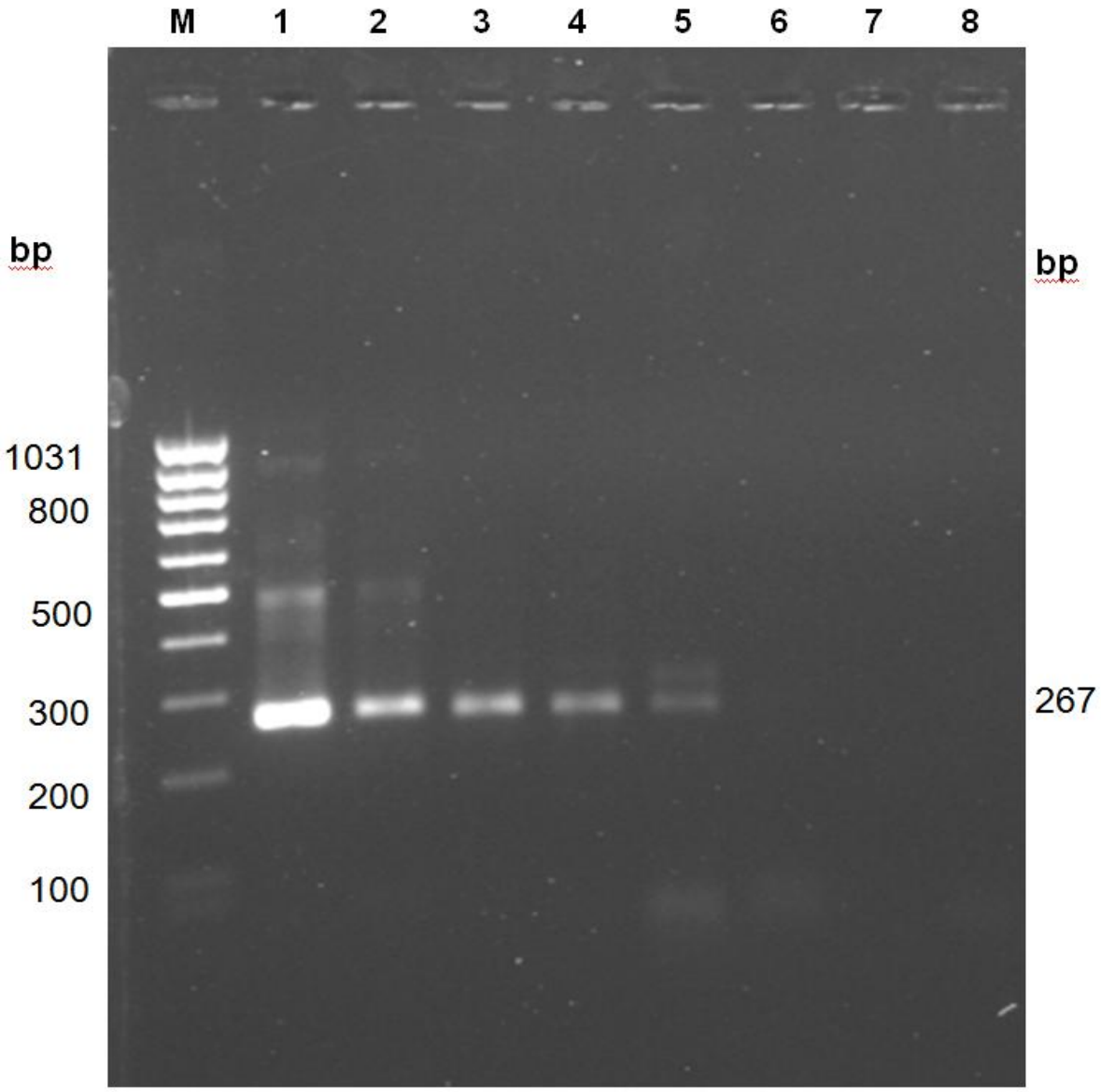

Fig. 6. PCR detection of *L. monocytogenes* in chicken meat by phenol extraction after enrichment. Lane M:100 bp DNA ladder; Lane 1: Spiking level 10^7 cfu·g^{-1}; Lane 2: Spiking level 10^6 cfu·g^{-1}; Lane 3: Spiking level 10^5 cfu·g^{-1}; Lane 4: Spiking level 10^4 cfu·g^{-1}; Lane 5: Spiking level 10^3 cfu·g^{-1}; Lane 6: Spiking level 10^2 cfu·g^{-1}; Lane 7: Spiking level 10 cfu·g^{-1}; Lane 8: Control.

Discussion

Conventional microbiological and immunological methods have their own limitations in detection of *L. monocytogenes* especially in terms of either specificity or sensitivity (Mattingly et al. 1988; Hitchins, 1998; Gangar et al. 2000). The PCR has been proven to be a specific and sensitive method for detection of this pathogen from different food samples including seafood and chicken (Levin, 2003).

Three different methods of processing of samples were compared in the PCR detection of *L. monocytogenes* from the freshwater fish, prawn and chicken meat. The purpose of using a two-step nested PCR was to increase both specificity and sensitivity. The results revealed that no amplified product was obtained from the DNA extracted by boiling lysis method. The reason may be due to the fact that the boiling lysis method cannot remove the inhibitory substances present in the samples, which lead to inhibition of the PCR assay. However, a 267 bp amplified product was obtained in the PCR using the template DNA extracted with phenol: chloroform: isoamyl alcohol method and the detection limits were found to be 10^4, 10^3 and 10^5 cfu·g^{-1} from fish, prawn and chicken meat respectively (Figs. 1, 2 and 3). This variation of sensitivity in fish, prawn and chicken meat may be due to the variation in complexity and level of PCR inhibitory substances present in fish, prawn and chicken meat. A similar observation was also reported by Wernars et al. (1991) in direct detection of *L. monocytogenes* by PCR from soft cheese. They indicated that processing of cheese samples by heating the homogenized suspension of cheese at various temperatures (40, 60 or 80 °C) did not yield any amplified product, but the extraction of homogenized suspension with phenol considerably decreased the PCR inhibition.

Enrichment before carrying out PCR has been found to yield a high level of sensitivity for the detection of *L. monocytogenes* (Duffy et al. 1999). Apart from high sensitivity, the method is also useful in distinguishing the dead and viable bacteria (Josephson et al. 1993). In this study, by employing enrichment before performing the PCR, the sensitivity of detection has been increased significantly as the detection limit was found to be 10^2, 10 and 10^3 cfu·g^{-1} in fish, prawn and chicken meat, respectively (Figs. 4, 5 and 6). This method can be recommended for detection of *L. monocytogenes* in place of conventional microbiological culture method, which takes at least a week to identify this pathogen.

Conclusion

This study indicates that the efficiency of nested PCR for detection of *L. monocytogenes* from freshwater fish, prawn and chicken meat depends on the DNA extraction method. Among the three methods of processing of samples used in this study, 'phenol extraction after enrichment' was found to be the most sensitive. Sample processing using the boiling lysis method is not suitable for detection of *L. monocytogenes* from fish, prawn and chicken meat by nested PCR.

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