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# Incidence of *Listeria* spp. in clinical and food samples in Mangalore, India

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**Abstract**

A prospective study was carried out in food and clinical samples from Mangalore, South India, during March 1997–December 2001, to determine the incidence of *Listeria* spp. Bacteriological investigations were done on 633 clinical and 320 food samples. *Listeria monocytogenes* was isolated from two clinical and two food samples. The incidence of *Listeria* spp. was 0.3% in clinical and 17.5% in food samples. Seafood, beef, raw milk, vegetables showed the presence of *Listeria innocua*.


*Keywords:* *Listeria* spp.; Food samples; India

## 1. Introduction

Ever since the foodborne nature of listeriosis was established (Schlech et al., 1983), there is increasing interest in understanding the risk associated with this organism in various foods. Although the incidence of the first human case of listeriosis was reported by Nyfeldt (1929), it is only since, 1981, after the three well-investigated listeriosis epidemics (one caused by coleslaw (Schlech et al., 1983), second caused by whole and 2% milk, Fleming et al. (1985)) and third caused by consumption of soft Mexican-style cheese (Linnan et al., 1988), that this organism came to be considered as a foodborne pathogen. The importance of food as a source of sporadic outbreaks of listeriosis is illustrated by the Center for Disease Control (Schuchat et al., 1992; Pinner et al., 1992). Vegetables, meat sausages, dairy products and seafood may be contaminated with *Listeria* spp. and are considered as a major source of infection. Moreover, *Listeria* spp. have a unique property of multiplication at 4°C in food stored in refrigerators. Hence, the risk of infection from a contaminated food increases after refrigeration (Hof et al., 1994).

Large majority of patients with listeriosis have an underlying condition which predisposes to infection by interfering with T cell-mediated immunity (Hof et al., 1998). At great risk are pregnant women and the unborn child, alcoholics, drug abusers, diabetics, patients receiving treatments which alters their natural immunity, AIDS patients, patients with malignancy and the elderly (WHO working group, 1988). Infection acquired early in pregnancy may lead to abortion, stillbirths or premature delivery. When the infection is acquired late in pregnancy, it can be transmitted transplacentally and lead to neonatal listeriosis. It may manifest at birth or late in neonatal period. Non-perinatal listeriosis is seen mainly in immuno-compromised adults and children. Typical overt listeriosis presents as sepsis and meningitis (Hof et al., 1998).

Although the presence of *L. monocytogenes* has been reported from a wide variety of foods, the incidence in tropical foods is very low (Jeyasekaran et al., 1996; Karunasagar and Karunasagar, 2000). In India, there are very few reports on the incidence of *Listeria monocytogenes* in clinical and food samples (Gupta et al., 1997; Thomas et al., 1981; Bhujwala and Hingorani, 1975; Bhujwala et al., 1974). Hence, the present study was undertaken to study the incidence of

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*L. monocytogenes* in various clinical samples from suspected cases of listeriosis, as well as food samples.

## 2. Methods

### 2.1. Samples collected

The clinical samples ($n = 633$) collected from various hospitals in Mangalore, India, during the period March 1997 to December 2001 included vaginal swabs, cervical swabs, aborted material, amniotic fluid, placental bit and fetal tissue obtained from febrile pregnant women with bad obstetric history like repeated abortions, intrauterine deaths, still births and preterm labor. cerebrospinal fluid (CSF) and blood samples were collected from neonates, elderly patients, cancer patients and HIV infected patients with suspected cases of meningitis and septicemia. Other clinical samples like pleural fluids, synovial fluids, peritonial and ascitic fluids and stool were also screened for the presence of *Listeria* spp. (Table 1). The food samples ($n = 320$) processed for the study were collected from retail outlets in Mangalore, India, during the period March 1997 to December 2001 included fresh, dry and smoked seafood, dairy products, fresh meat and vegetable samples (Tables 2 and 3).

### 2.2. Isolation of Listeria spp.

Non-sterile site clinical samples like tissues, placental bits, vaginal and cervical swabs were processed by United States Department of Agriculture (USDA) and Netherlands Government Food Inspection Service (NGFIS) methods and also by cold enrichment methods (Swaminathan et al., 1995). Briefly, 25 g of the sample was homogenized in 225 ml of Pre Enrichment Broth (PEB) (Tryptic Soy broth with 0.6% yeast extract). After incubating for 24 h at 30°C, 10 ml of PEB was transferred to 90 ml of University of Vermont Medium (UVM) I and incubated for 24 h at 30°C. 0.1 ml of UVM I was inoculated to UVM II and also streaked on Oxford agar and Polymixin Acryflavin Lithium chloride Ceftazidime Asculin Mannitol (PALCAM) agar plates. UVM II was incubated at 37°C for 24 h and then streaked on to Oxford and PALCAM agar plates. Agar plates were incubated at 37°C for 48 h. In cold enrichment procedure, 10 g of sample/swab was transferred to 50 ml of brain–heart infusion (BHI) broth and incubated at 4°C for 6 weeks. The enrichment was streaked on Oxford, PALCAM and Blood agar plates, at weekly intervals. Clinical samples like blood, CSF, amniotic fluid and other body fluids were collected in BHI broth or in sterile plain bottles, depending on the samples and cultured on 5% sheep blood agar.

The procedure adopted for the isolation of *Listeria* spp. from food samples was a modification of USDA (McClain and Lee, 1988) and FDA (Lovett, 1988) methods. Briefly, 25 g of the sample was homogenized in 225 ml PEB and incubated at 30°C for 24 h. PEB sample (10 ml) was transferred to 90 ml of UVM I and incubated at 30°C for 24 h. UVM I (0.1 ml) was transferred to 10 ml of Modified Fraser broth (McClain and Lee, 1989) and incubated at 37°C. At 24 h, 4 days and 7 days the samples were streaked on Oxford agar (Curtis et al., 1989) and PALCAM agar (Van Netten et al., 1989) plates and incubated at 37°C for 48 h.

Table 1
Incidence of *Listeria* spp. in clinical samples

| Samples | Source | | | | | Total | Number positive for *L. monocytogenes* |
|---|---|---|---|---|---|---|---|
| | Pregnant women | Neonates | Elderly patients | Cancer patients | HIV patients | | |
| Aborted material | 21 | — | — | — | — | 21 | — |
| Amniotic fluid | 01 | — | — | — | — | 01 | — |
| Ascitic fluid | — | — | 20 | — | — | 20 | — |
| Blood | 06 | 78 | 48 | 12 | 16 | 160 | — |
| Cervical swabs | 69 | — | — | — | — | 69 | — |
| CSF | — | 103 | 89 | 09 | 13 | 214 | — |
| Fetal tissue | 12 | — | — | — | — | 12 | — |
| Gastric aspirate | — | 01 | — | — | — | 01 | — |
| Peritonial fluid | — | — | 05 | — | — | 05 | — |
| Placental bit | 65 | — | — | — | — | 65 | 02 (3.1%) |
| Pleural fluid | — | 07 | 26 | 04 | — | 37 | — |
| Stool | — | — | 06 | — | — | 06 | — |
| Synovial fluid | — | — | 02 | — | — | 02 | — |
| Umbilical cord | — | 01 | — | — | — | 01 | — |
| Vaginal swabs | 19 | — | — | — | — | 19 | — |
| Total | 193 | 190 | 196 | 25 | 29 | 633 | 02 (0.3%) |

Table 2
Incidence of *Listeria* spp. in seafood

| Sample | Number tested | Number positive for *Listeria* spp. | |
|---|---|---|---|
| | | *L. innocua* | *L. monocytogenes* |
| Lactarius | 10 | 05 (50%) | — |
| Mackerels | 14 | 01 (7.1%) | — |
| Crab | 14 | 11 (78.6%) | — |
| Croakers | 11 | 06 (54.5%) | — |
| Pomfret | 12 | 02 (16.7%) | — |
| Fresh prawn | 11 | 01 (9.1%) | — |
| Dry prawn | 27 | 03 (11.1%) | — |
| Sardines | 15 | 05 (33.3%) | — |
| Fresh flat fish | 35 | 13 (37.1%) | 01 (2.9%) |
| Dry flat fish | 15 | — | — |
| Clams | 24 | 01 (4.2%) | 01 (4.2%) |
| Smoked tuna | 22 | — | — |
| Total | 210 | 48 (22.9%) | 02 (0.95%) |

Table 3
Incidence of *Listeria* spp. in other food samples

| Sample | Number tested | Number positive for *Listeria* spp. | |
|---|---|---|---|
| | | *L. innocua* | *L. monocytogenes* |
| Cheese | 12 | — | — |
| Ice-cream | 09 | — | — |
| Raw milk | 35 | 01 (2.9%) | — |
| Pasteurized milk | 05 | — | — |
| Beef | 10 | 01 (10%) | — |
| Chicken | 09 | — | — |
| Mutton | 05 | — | — |
| Cabbage | 05 | — | — |
| Coriander leaves | 10 | 03 (30%) | — |
| Palak leaves | 10 | 01 (10%) | — |
| Total | 110 | 06 (5.5%) | — |

### 2.3. Identification of Listeria spp.

Grey green colonies with black sunken centers from PALCAM agar plates and black colonies with black sunken centers from Oxford agar, suspected to be *Listeria* spp., were picked up and cultured on tryptic soy agar with 0.6% yeast extract. All the isolates were subjected to standard biochemical tests such as catalase test, motility at 25°C and 37°C, acid production from glucose, mannitol, rhamnose, xylose and α-methyl D-mannoside, nitrate reduction, hydrolysis of esculin, methyl red test and Voges-Proskauer test. The isolates were identified based on the criteria suggested by Lovett (Lovett, 1988) and Jones and Seeliger (1992). *L. monocytogenes* isolates identified biochemically were tested for hemolytic activity by blood agar plate method (McKellar, 1994) and also by micro-titer plate method after growing the cultures in charcoal treated proteose peptone broth (Dominguez-Rodriguez et al., 1986).

### 2.4. Antibiotic sensitivity test

Antibiotic susceptibility patterns of all the *Listeria* isolates confirmed biochemically were performed by standard disc diffusion method (Bauer et al., 1966). Antibiotic discs used were ampicillin, chloramphenicol, ciprofloxacin, cotrimoxazole, erythromycin, gentamycin and penicillin. The results were interpreted as per the NCCLS criteria (National Committee for Clinical Laboratory Standards, 1993).

### 2.5. Serotyping

All the isolates of *L. monocytogenes* were serotyped at the University of Manheim, Germany.

### 2.6. Confirmation of L. monocytogenes by ELISA and PCR

All the isolates of *Listeria* spp. confirmed biochemically were subjected to enzyme linked immuno-sorbent assay (ELISA) using PATHALERT *Listeria* ELISA Kit (marketed by Merck, Germany) which detects *Listeria* antigen p60. The *L. monocytogenes* isolates confirmed by ELISA were subjected to polymerase chain reaction (PCR) using the following primer pairs described by Bubert et al. (1992): UnilisA–Lis1B, MonoA–MonoB and UnilisA–MonoB. Briefly, a loop full of culture was suspended in 100 µl of sterile distilled water in a PCR tube and heated at 100°C for 15 min in a dry bath. This served as crude DNA extract. The PCR conditions were as described by Bubert et al. (1992).

### 2.7. Demonstration of the invasiveness of L. monocytogenes isolated from food and clinical samples

Two isolates of *L. monocytogenes* from food samples and two from clinical samples along with *L. monocytogenes* EGD wild type strain were studied for their invasive properties using human colon carcinoma cell line Caco-2 (ECACC 86010202). Caco-2 cell line was cultured in minimal essential medium (GIBCO) supplemented with 10% fetal calf serum 2 mM L-glutamine, 1% non-essential amino acids, penicillin (100 U $ml^{-1}$) and streptomycin (100 µg $ml^{-1}$) in a 5% $CO_2$ incubator.

The method described by Gaillard et al. (1987) was used for cell infection. At 24 h before infection, Caco-2 cells were trypsinized and the cell concentration was adjusted to $2 \times 10^5$ $ml^{-1}$. One-milliliter portion of cell suspension in medium without penicillin and streptomycin was seeded out in each well of a 24-well tissue culture plate. Bacterial cultures grown for 18 h at 37°C were centrifuged, washed in phosphate buffered saline (PBS) and suspended in tissue culture medium without supplements to obtain a concentration of about $10^6$ cfu per ml. Host cells were washed with PBS containing

calcium and magnesium [PBS (Ca, Mg)]. The bacterial suspension (1 ml) were added to each well in tissue culture plates and the plates were centrifuged at 500*g* for 10 min at room temperature and incubated at 37°C. The bacterial numbers in the suspension were enumerated on BHI agar. At 1 h (this was considered the invasion period), the cells were washed three times with PBS (Ca, Mg) and 1 ml of complete medium without penicillin and streptomycin but containing gentamicin at a concentration of 10 μg ml$^{-1}$ was added to each well. According to Gaillard et al. (1987), gentamicin at 5 μg ml$^{-1}$ would kill extracellular *L. monocytogenes* but intracellular bacteria would not be affected because of lesser penetration of gentamicin into eukaryotic (Caco-2) cells. To study invasion, 1 h after the addition of gentamicin the cells were washed 3 times with PBS (Ca, Mg) and lysed by addition of 1 ml distilled water. Tenfold dilutions were plated on BHI agar plates. This experiment was repeated thrice, each time in duplicate, and average result of the typical experiment is shown in Table 5.

## 3. Results and discussion

*L. monocytogenes* was isolated from 3.1% of the placental bits screened from pregnant women with bad obstetric history (Table 1). All other clinical samples from pregnant women and other categories of patients were negative for *Listeria* spp. Earlier studies from India indicated the incidence rate of *L. monocytogenes* in pregnant women, with bad obstetric history, to be in the vicinity of 1.34% and 4% (Bhujwala and Hingorani, 1975; Stephen et al., 1978). Incidence of neonatal listeriosis in India was reported by Thomas et al. (1981). Gupta et al. (1997) reported the isolation of *L. monocytogenes* from blood samples (3 cases). However, *L. monocytogenes* was not isolated from any of the CSF samples investigated by Bhujwala et al. (1974).

The incidence of *L. monocytogenes* in clinical samples noted in this study is similar to that reported by others in India. The study suggests that abortion is the most common form of listeriosis in this part of India. The observation that *L. monocytogenes* could not be isolated from 78 blood samples and 103 CSF samples in neonates (Table 1) suggests that this organism is rarely involved in septicemia and meningitis in neonates in this part of India. Similar results were obtained with samples from elderly, cancer patients and HIV infected case.

*L. innocua* were found in 30.8% and *L. monocytogenes* in 1.3% of fresh raw fish samples (Table 2). Other species of *Listeria* were not isolated in this study. *L. monocytogenes* was isolated from 4.2% of raw clams and 2.9% of raw flat fish. In the present study, the *L. monocytogenes* isolate from clams belonged to serovar 1 and that from flat fish belonged to serovar 4. Serotypes of seafood associated *L. monocytogenes* have been investigated by very few workers. Wong et al. (1990), Rorvik and Yndestad (1991), Boerlin et al. (1997) and Inoue et al. (2000) noted that majority of the isolates of *L. monocytogenes* from seafood belonged to serotypes 1 and 4. A similar observation has been made in the present study too.

Smoked fish samples were free from *Listeria* spp. Earlier reports suggested the absence of *L. monocytogenes* in tropical fish (Fuchs and Surendran, 1989; Manoj et al., 1991; Karunasagar et al., 1992; Kamat and Nair, 1994). However, Jeyasekaran et al. (1996) reported the incidence of various *Listeria* spp. including *L. monocytogenes* in a variety of fish samples. In the present study, the incidence of *L. monocytogenes* in seafoods is 0.95%. Baek et al. (2000) reported the absence of *L. monocytogenes* in dried seafood in Korea.

It is interesting to note that among all food samples studied, highest incidence of *L. innocua* was observed in seafood. *L. monocytogenes* was also isolated only from seafood. This suggests that the risk of acquiring listeriosis is higher through seafood in India. Samples that were positive for *L. monocytogenes* were raw seafood which could be cooked before consumption. Nevertheless, presence of this organism in raw seafood poses a health risk in kitchen where raw and cooked seafood may be stored and handled.

Raw meat samples analysed included chicken, beef and mutton. Ten percent of the beef samples processed showed the presence of only *L. innocua*. Chicken and mutton samples were free from *Listeria* spp. (Table 3). Varying incidence rates of *L. monocytogenes* were reported in meat and meat products by different workers (Baek et al., 2000; Farber et al., 1989; Arumugaswamy et al., 1994; Ojeniyi et al., 1996).

*L. innocua* was isolated from 10% of the palak leaves and 30% of coriander leaf samples (Table 3). Varying incidence rates of *L. monocytogenes* in raw vegetables were reported (Arumugaswamy et al., 1994; Uyttendale et al., 1999). However, Farber et al. (1989) and Kaneko et al. (1999) reported the absence of *L. monocytogenes* from vegetable samples analysed. The results of the present study are in line with these observations.

Among the dairy products analysed, only raw milk showed the presence of *L. innocua* (2.9%) (Table 3). Incidence rates of *L. monocytogenes* from 0.94% to 15% was reported by Harvey and Gilmour (1992), Uraz and Yucel (1999) and Baek et al. (2000) in raw milk samples. In Ice cream samples, very low incidence rate was reported by Farber et al. (1989) and quite a high rate of 6.1% was reported by Baek et al. (2000). In the present study, the dairy products were free from *L. monocytogenes*.

In the present study, antibiotic susceptibility pattern showed that all the isolates of *L. monocytogenes* were susceptible to the antibiotics tested. However, few of the

Table 4
Antibiotic susceptibility patterns of typical and atypical isolates of *Listeria*

| Antibiotic disc | *L. innocua* ($n = 54$) | | | *L. monocytogenes* ($n = 04$) | | | Atypical isolates ($n = 05$) | | |
|---|---|---|---|---|---|---|---|---|---|
| | S | I | R | S | I | R | S | I | R |
| Ampicillin (10 μg) | 54 | 0 | 0 | 04 | 0 | 0 | 04 | 0 | 01 |
| Ciprofloxacin (5 μg) | 36 | 18 | 0 | 04 | 0 | 0 | 05 | 0 | 0 |
| Cotrimoxazole (25 μg) | 39 | 01 | 14 | 04 | 0 | 0 | 02 | 0 | 03 |
| Erythromycin (15 μg) | 23 | 22 | 09 | 04 | 0 | 0 | 01 | 04 | 0 |
| Gentamycin (10 μg) | 54 | 0 | 0 | 04 | 0 | 0 | 05 | 0 | 0 |
| Penicillin (10 units) | 54 | 0 | 0 | 04 | 0 | 0 | 03 | 0 | 02 |
| Chloramphenicol (30 μg) | 54 | 0 | 0 | 04 | 0 | 0 | 05 | 0 | 0 |

S: Sensitive.
I: Intermediate.
R: Resistant.

*L. innocua* isolates were resistant to erythromycin and cotrimoxazole (Table 4).

Resistance of *L. monocytogenes* to penicillin and gentamycin, the drugs of choice for treatment of listeriosis, have not been reported (Charpentier and Courvalin, 1999). A similar observation has been made in our study.

All the *L. monocytogenes* strains as well as the *L. monocytogenes* EGD strain were able to invade Caco-2 cell lines. Average result of the typical experiment is shown in Table 5. It was interesting to note that, isolates of *L. monocytogenes* from flat fish and one strain from human placental tissue were more invasive to Caco-2 cell lines. Among these two, the isolate from flat fish appeared to be most invasive. Occurrence of such invasive strains in food samples warrants strict precautionary measures.

In this study, there were five atypical isolates: three from placenta and two from coriander leaves. These atypical isolates showed all the characteristics similar to *L. monocytogenes* except α methyl D-mannoside fermentation. Hence the biochemically typical and atypical isolates of *Listeria* spp. were subjected to ELISA and PCR. All the biochemically confirmed isolates showed a positive reaction in PATHALERT *Listeria* ELISA (Merck Diagnostics, Germany) and all the ELISA Positive *L. monocytogenes* isolates were positive by PCR too. Atypical isolates were negative by ELISA and PCR. The amplification product of 1.5 kb was seen for genus-specific reaction with primers UnilisA and Lis1B (Fig. 1) and 0.4 and 0.5 kb were seen for *Listeria*-species-specific reaction with primers MonoA MonoB and UnilisA MonoB respectively (Figs. 2 and 3). These results show that ELISA and PCR are important in confirming the identity of biochemically atypical strains, which otherwise would have been misidentified as *L. monocytogenes*. Our study shows a very low incidence rate of *L. monocytogenes* in clinical as well as food samples, which supports the findings that listeriosis is an uncommon infection and has unique predilection for pregnant women (Silver, 1998).

Table 5
Invasion of Caco-2 cells by clinical and fish isolates of *L. monocytogenes* (values are average of duplicates in a typical experiment that was repeated thrice)

| Bacterial strains | Number of bacteria per well | | |
|---|---|---|---|
| | Infection | Invasion | % Invasion |
| *L. monocytogenes* EGD | $1.5 \times 10^6$ | $1.2 \times 10^4$ | 0.8 |
| *L. monocytogenes* (placenta) | $1.3 \times 10^6$ | $4.0 \times 10^4$ | 3.1 |
| *L. monocytogenes* (placenta) | $2.1 \times 10^6$ | $1.1 \times 10^4$ | 0.5 |
| *L. monocytogenes* (flat fish) | $1.9 \times 10^6$ | $5.9 \times 10^4$ | 3.1 |
| *L. monocytogenes* (clams) | $1.4 \times 10^6$ | $2.0 \times 10^4$ | 1.4 |

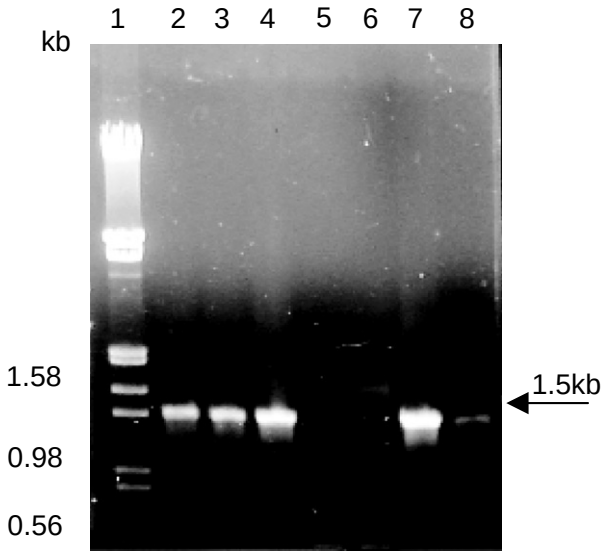


Fig. 1. Genus-specific identification of *Listeria* spp. by PCR with primer pairs UnilisA-Lis1B. Lanes 1 molecular weight marker (Lambda DNA/Eco RI-Hind III Double digest); 2, *L. monocytogenes* (NCTC 7973); 3–4, isolates from placenta; 5–6, atypical isolates; 7, isolate from clams; 8, isolate from flat fish.

It has been suggested that *L. monocytogenes* and *L. innocua* share the same ecological niche and therefore *L. innocua* could be used as indicator strain for the presence of *L. monocytogenes* (King et al., 1990). The

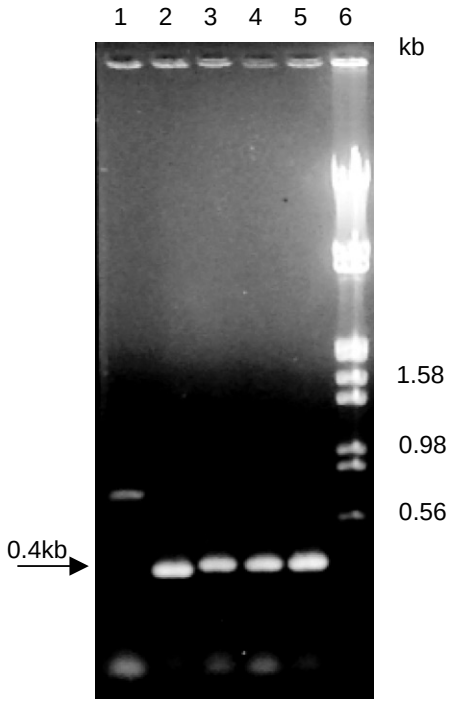


Fig. 2. Species-specific identification of *Listeria monocytogenes* by PCR with primer pairs MonoA-MonoB. Lanes: 1, isolate from flat fish; 2, isolate from clams; 3–4, isolates from placenta; 5, *L. monocytogenes* (NCTC 7973); 6, molecular weight marker (Lambda DNA/Eco RI-Hind III Double digest).

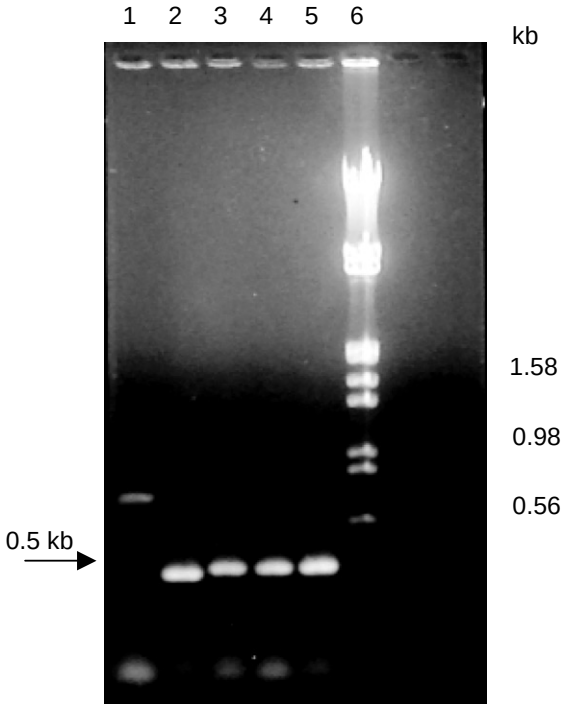


Fig. 3. Species-specific identification of *Listeria monocytogenes* by PCR with primer pairs UnilisA-MonoB. Lanes 1 isolate from flat fish; 2, isolate from clams; 3–4, isolate from placenta; 5, *L. monocytogenes* (NCTC 7973); 6, molecular weight marker (Lambda DNA/Eco RI-Hind III Double digest).

observation that incidence of *L. monocytogenes* could be detected only in seafood which showed over, 20% incidence of *L. innocua* supports this hypothesis.

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