Indian J Med Res 117, January 2003, pp 19-24

Typing of *Listeria monocytogenes* isolates by random amplification of polymorphic DNA

B. Dhanashree*, S.K. Otta, I. Karunasagar & I. Karunasagar

**Department of Microbiology, Kasturba Medical College & Department of Microbiology, University of Agricultural Sciences, College of Fisheries, Mangalore, India*

Received August 23, 2002

Background & objectives: *Listeria monocytogenes* **is an important food-borne pathogen causing meningitis and septicaemia in newborns and immunocompromised persons, abortion and preterm labour in pregnant women. Though various methods are available for typing *L. monocytogenes*, RAPD analysis has been used for epidemiological purposes in developed countries due to its greater discriminating ability. However, as there are no published reports from India on the typing of *L. monocytogenes* by RAPD technique the present study was undertaken to type isolates of *L. monocytogenes* from clinical, food and veterinary samples.**

Methods: **Isolates of** *L. monocytogenes* **were subjected to RAPD using four decamer random primers R_1, R_2, R_3 and R_4. Amplified products were analysed by agarose gel electrophoresis.**

Results: **Eight strains of** *L. monocytogenes* **on RAPD analysis generated 4 distinct profiles each with R_1 and R_4 primers and 3 different profiles with R_2 and R_3 primers. The isolates from fish, clinical and veterinary samples showed different profiles with respect to each other. Isolate from flat fish (serovar 4) showed a different profile from that of clams (serovar 1). Two isolates from placenta (serovar 1) showed similar profiles and all the isolates from veterinary samples generated similar profiles.**

Interpretation & conclusion: **RAPD analysis in the present study allowed discrimination of isolates among the same serotype but from different sources. Since RAPD is a rapid technique and offers greater discrimination of strains, this method may be used for typing** *L. monocytogenes* **in India.**

Key words *Listeria monocytogenes* - random amplification of polymorphic DNA (RAPD) - typing

A number of outbreaks of food-borne listeriosis have occurred in many countries, since the early 1980s[1-3]. It is now known that, consumption of contaminated food is the primary vehicle of transmission of listeriosis[4,5]. *Listeria* spp. have been isolated from a variety of raw and processed food, but the main source of contamination is unknown. In the natural environment, several serotypes of *Listeria monocytogenes* are known to exist but generally clinical isolates belong to 1/2a and 4b. Serotyping of *Listeria* is difficult and expensive due to non availability of commercial antisera. Therefore, alternate typing methods are essential to differentiate clinical and environmental strains. Typing methods may serve to identify or trace those *Listeria* clones, and their environmental niches, that are the greatest threat to mankind[6].

During the last decade, the application of PCR based methods has increased in the fields of medicine, food and environmental microbiology. An important promising application of PCR is the epidemiological typing of microbial isolates, by a technique known as random amplification of polymorphic DNA [RAPD or arbitrary primer PCR (AP-PCR)][7]. A protocol for epidemiological typing of *Listeria* spp. by RAPD has been previously described[8]. The usefulness of RAPD-analysis for the epidemiological study of *L. monocytogenes* isolates has

been confirmed by other groups[9-15]. However, no such studies have been reported from India. Hence, in the present study RAPD was used for the typing of *L. monocytogenes* isolates from clinical, food and veterinary samples collected from Mangalore, India.

Material & Methods

Samples: The clinical samples (n=633) collected from various hospitals in Mangalore, India during March 1997 to December 2001 included vaginal swabs (19), cervical swabs (69), material obtained after incomplete abortion (21), amniotic fluid (1), placental bits (65) and foetal tissue (12) obtained from febrile pregnant women with bad obstetric history like repeated abortions, intrauterine deaths, still births and preterm labour. Cerebrospinal fluid (214) and blood samples (160) were collected from neonates, elderly patients, cancer patients and HIV infected patients suspected to have meningitis or septicaemia. Other clinical samples like pleural fluids (37), synovial fluids (2), peritonial (5) and ascitic fluids (20) and stool (8) were also screened for the presence of *Listeria* spp. The food samples (n=320) processed for the study were collected from retail outlets in Mangalore, India during the same period and included fresh, dry and smoked seafood, dairy products, fresh meat and vegetables.

Isolation of Listeria spp.: Clinical samples like tissues, placental bits, vaginal and cervical swabs from non sterile sites were processed by the methods of the United States Department of Agriculture (USDA) and Netherlands Government Food Inspection Service (NGFIS) and also by cold enrichment method[16]. Briefly, 25 g of sample was homogenized in 225 ml of pre enrichment broth (PEB, Hi-Media Laboratories, Mumbai) [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 the University of Vermont Medium I (UVM I) (Hi-Media Laboratories, Mumbai) and incubated for 24 h at 30°C. 0.1 ml of UVM I was inoculated to UVM II (Hi-Media Laboratories, Mumbai) and also streaked on Oxford agar (Hi-Media Laboratories, Mumbai) and polymixin acryflavin lithium chloride ceftazidime asculin mannitol (PALCAM, Hi-Media Laboratories, Mumbai) agar plates. UVM II was incubated at 37°C for 24 h and then streaked on to Oxford and PALCAM agar plates. All the agar plates were incubated at 37°C for 48 h. In the cold enrichment procedure, 10 g of sample/swab was transferred to 50 ml brain heart infusion (BHI, Hi-Media Laboratories, Mumbai) broth and incubated at 4°C for six wk. 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 per cent sheep blood agar.

The procedure adopted for the isolation of *Listeria* spp. from food samples was a modification of USDA[17] and FDA[18] methods. Briefly, 25 g sample was homogenized in 225 ml PEB and incubated at 30°C for 24 h. 10 ml of PEB sample was transferred to 90 ml of UVM I and incubated at 30°C for 24 h 0.1 ml of UVM I was transferred to 10 ml of modified Fraser broth[19] (Hi-Media Laboratories, Mumbai) and incubated at 37°C. At 24 h, 4 days and 7 days the samples were streaked on Oxford agar[20] and PALCAM agar[21] plates and incubated at 37°C for 48 h.

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 per cent yeast extract. All the isolates were subjected to standard biochemical tests such as catalase test, motility at 25 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 (reagents for biochemical tests were procured from HI-Media Laboratories, Mumbai). The isolates were identified based on the criteria suggested by Lovett[18] and Jones and Seeliger[22]. Biochemically identified *L. monocytogenes* isolates were tested for haemolytic activity by

blood agar plate method[23] and also by micro titre plate method after growing the cultures in charcoal treated proteose peptone broth[24].

Bacterial strains: Four strains of *L. monocytogenes* were isolated from the clinical and food samples processed, two from human clinical samples (placenta) and two from food samples (one each from clams and flat fish). Four veterinary strains of *L. monocytogenes* isolated from sheep suffering from encephalitis were provided by the Haryana Agricultural University, Hissar, India. A standard strain of *L. monocytogenes* (NCTC 7973, PHLS Central Public Health Laboratory, London) was also used in the study.

Preparation of DNA: *L. monocytogenes* strains were grown on BHI agar plates for 24 h at 30°C. A single colony was transferred to BHI broth (1 ml) and grown for 18 h at 37°C. Cells were pelleted. Pellets were washed thrice with sterile saline and suspended in 50 µl of sterile distilled water. This was heated at 95°C for 10 min in a dry bath (Bangalore Genei Pvt. Ltd., Bangalore) followed by cooling at 4°C. This served as crude DNA lysate. From this lysate a 100 fold dilution was prepared by adding 10 µl of lysate to 990 µ of sterile distilled water and used as a source of DNA in amplification reaction. Four custom synthesized decamer random primers[25] (Bangalore Genei Pvt. Ltd., Bangalore) R_1 (5'-CCGCAGCCAA3'), R_2 (5'-GCGATCCCCA-3'), R_3 (5'-AAGAGCCCGT-3') and R_4 (5'-GTGGATGCGA-3') were used at a concentration of 0.5 µg/reaction.

The polymerase chain reaction (PCR) mixture contained the following reagents (Bangalore Genei Pvt. Ltd., Bangalore) in the PCR tube: 41.25µl sterile distilled water, 5µl assay buffer for Taq DNA polymerase, 1µl dNTP mix, 0.75µl Taq DNA polymerase, 1 µl random primer and 1 µl of crude DNA extract. The PCR tubes were heated in DNA thermocycler (PTC-100; MJ Research, Watertown, MA, USA). PCR reaction was carried out upto 35 cycles. The reaction conditions were initial delay at 94°C for 5 min, denaturation at 94°C for 1 min, annealing at 36°C for 1 min and polymerization at 72°C for 2 min and final delay at 72°C for 10 min. The amplified products were resolved by electrophoresis on 1 per cent agarose gel in IX TAE buffer (Bangalore Genei Pvt. Ltd., Bangalore) containing 0.5 µg of ethidium bromide per ml[26]. Lambda DNA/Eco-R_1-Hind III double digest was used as molecular weight marker (Bangalore Genei Pvt. Ltd.). The gels were photographed under UV transillumination. Banding profiles were determined and arbitrarily numbered. The method used to produce the cell lysate as a source of DNA in the amplification protocol was standardized and this allowed a reproducible pattern to be obtained in RAPD. Serotyping of the clinical and food isolates was done at the University of Manheim, Germany. However, serotyping of the veterinary isolates was not done.

Results & Discussion

Eight strains of *L. monocytogenes* on RAPD analysis, using 4 random primers, generated 4 profiles each with R_1 and R_4 primers (Figs 1 and 2) and 3 profiles with R_2 and R_3 (Figs 3 and 4). Among the 2 strains isolated from food, the isolate from flat fish belonging to serovar 4 (Lane 1) showed a different profile when compared to the isolate from clams belonging to serovar 1 (Lane 2). Thus isolate belonging to serovar 4 showed different profile with all primers used when compared to the isolates belonging to serovar 1 (Figs 1-4). The strains belonging to serovar 1 isolated from different sources (clams and placenta) showed different profiles with R_1 and R_4 primers, but identical profiles in major bands with R_2 and R_3 primers. Thus using R_1 and R_4 primers discrimination could be made among clams and placental isolates belonging to the same serovar. All the veterinary isolates showed identical profiles with the 4 random primers (Lanes 5-8). However, serotype of the veterinary isolate was not available to compare their RAPD results with serotyping.

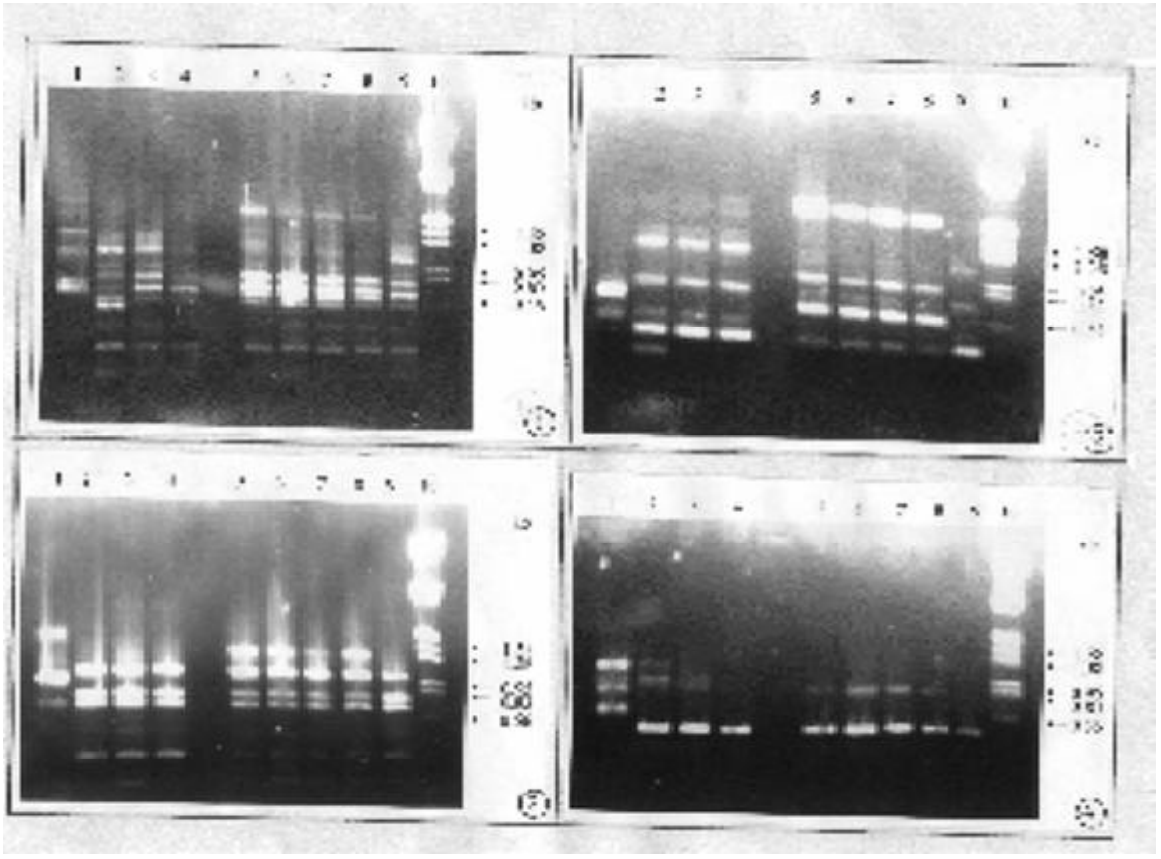

Fig. 1. RAPD typing of *L. monocytogenes* isolates using R_1 primer. Lanes **1**, isolate from flat fish; **2**, isolate from clams; **3-4**, isolates from placenta; **5-8**, isolates from veterinary samples; **9**, *L. monocytogenes* (NCTC 7973); **10**, molecular weight marker (Lambda DNA/Eco RI-Hind III double digest). **Fig. 2.** RAPD typing of *L. monocytogenes* isolates using R_4 primer. Lanes **1**, isolate from flat fish; **2**, isolate from clams; **3-4**, isolates from placenta; **5-8**, isolates from veterinary samples; **9**, *L. monocytogenes* (NCTC 7973); **10**, molecular weight marker (Lambda DNA/Eco RI-Hind III double digest). **Fig. 3.** RAPD typing of *L. monocytogenes* isolates using R_2 primer. Lanes **1**, isolate from flat fish; **2**, isolate from clams; **3-4**, isolates from placenta; **5-8**, isolates from veterinary samples; **9**, *L. monocytogenes* (NCTC 7973); **10**, molecular weight marker (Lambda DNA/Eco RI-Hind III double digest). **Fig. 4.** RAPD typing of *L. monocytogenes* isolates using R_3 primer. Lanes **1**, isolate from flat fish; **2**, isolate from clams; **3-4**, isolates from placenta; **5-8**, isolates from veterinary samples; **9**, *L. monocytogenes* (NCTC 7973); **10**, molecular weight marker (Lambda DNA/Eco RI-Hind III double digest).

Earlier workers have shown that RAPD is a rapid and simple technique which was used to analyze the relationship among strains from epidemics associated with milk products, sporadic listeriosis and field strains from dairy products and the environment[27,28]. RAPD technique has also been used for typing of epidemiologically unrelated strains[29]. Previous studies[12,27-29] have also shown that RAPD analysis allows discrimination among isolates of the same serotype and also among isolates from a common source though identical profiles were obtained for some isolates belonging to the same serovar. Our results are in agreement with these earlier findings[12,27-29] as primers R_1 and R_4 distinguish between food and clinical isolates belonging to serovar 1. However, primers R_2 and R_3 show identical profiles for the isolates belonging to serovar 1 irrespective of the source from which they were isolated. Differentiation between isolates belonging to serovars 1 and 4 could be easily made with all 4 primers and all the veterinary isolates could be discriminated from the food and clinical isolates in this study.

Though a fewer number of isolates was used in the present study, it is interesting to note that food, clinical and veterinary samples showed different RAPD profiles with respect to each other. RAPD analysis in our study allowed discrimination of isolates among the same serotype but from different sources. Hence, the technique could be used for typing of *L. monocytogenes* strains.

Acknowledgment

The authors thank Dr Usha Mandhokot, Department of Veterinary Public Health and Epidemiology, Haryana Agricultural University, Hissar, India for providing the veterinary isolates used in the study. Authors also thank Prof. Hof, University of Manheim, Germany for serotyping the clinical and food isolates.

References

1. Schlech WF 3rd, Lavigne PM, Bortolussi RA, Allen AC, Haldane EV, Wort AJ, *et al*. Epidemic listeriosis - evidence for transmission by food. *N Engl J Med* 1983; *308* : 203-6.
2. Fleming DW, Cochi SL, MacDonald KL, Brondum J, Hayes PS, Plikaytis BD, *et al*. Pasteurized milk as a vehicle of infection in an outbreak of listeriosis. *N Engl J Med* 1985; *312* : 404-7.
3. Farber JM, Peterkin PI. *Listeria monocytogenes*, a food-borne pathogen. *Microbiol Rev* 1991; *55* : 476-511.
4. Rocourt J, Cossart P. *Listeria monocytogenes*. In : Doyle MP, Beuchat LR, Montville J, editors. *Food microbiology*: *fundamentals and frontiers*. Washington DC: American Society for Microbiology Press; 1997 p. 337-52.
5. McLauchlin J. The pathogenicity of *Listeria monocytogenes*: a public health perspective. *Rev Med Microbiol* 1997; *8* : 1-14.
6. Slade PJ. Monitoring *Listeria* in the food production environment III. Typing methodology. *Food Res Int* 1992; *25* : 215-25.
7. Williams JG, Kubelik AR, Livak KJ, Rafalski JA, Tingey SV. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res* 1990; *18* : 6531-5.
8. Mazurier SI, Wernars K. Typing of *Listeria* strains by random amplification of polymorphic DNA. *Res Microbiol* 1992; *143* : 499-505.
9. Boerlin P, Bannerman E, Ischer F, Rocourt J, Bille J. Typing *Listeria monocytogenes*: a comparison of random amplification of polymorphic DNA with 5 other methods. *Res Microbiol* 1995; *146* : 35-49.
10. Czajka J, Batt CA. Verification of casual relationships between *Listeria monocytogenes* isolates implicated in food-borne outbreaks of listeriosis by randomly amplified polymorphic DNA patterns. *J Clin Microbiol* 1994; *32* : 1280-7.
11. Farber JM, Addison CJ. RAPD typing for distinguishing species and strains in the genus *Listeria. J Appl Bacteriol* 1994; *77* : 242-50.
12. Lawrence LM, Harvey J, Gilmour A. Development of a random amplification of polymorphic DNA typing method for *Listeria monocytogenes. Appl Environ Microbiol* 1993; *59* : 3117-9.
13. MacGowan AP, O'Donaghue K, Nicholls S, McLauchlin J, Bennett PM, Reeves DS. Typing of *Listeria* spp. by random amplified polymorphic DNA (RAPD) analysis. *J Med Microbiol* 1993; *38* : 322-7.
14. Niederhauser C, Hofelein C, Allmann M, Burkhalter P, Luthy J, Candrian U. Random amplification of polymorphic bacterial DNA: evaluation of 11 oligonucleotides and application to food contaminated with *Listeria monocytogenes*. *J Appl Bacteriol* 1994; *77* : 574-82.
15. Destro MT, Leitao MFF, Farber JM. Use of molecular typing methods to trace the dissemination of *Listeria monocytogenes* in a shrimp processing plant. *Appl Environ Microbiol* 1996; *62* : 705-11.
16. Swaminathan B, Rocourt J, Bille J. *Listeria.* In: Murray PR, Baron EJ, Pfaller MA, Tenover FC, Yolken RH, editors. *Manual of clinical microbiology*, 6th ed. Washington DC: ASM Press; 1995 p. 341-8.
17. McClain D, Lee WH. Development of USDA-FSIS method for isolation of *Listeria monocytogenes* from raw meat and poultry. *J Assoc Off Anal Chem* 1988; *71* : 660-4.
18. Lovett J. Isolation and identification of *Listeria monocytogenes* in dairy products. *J Assoc Off Anal Chem* 1988; *71* : 658-60.
19. McClain D, Lee WH. FSIS method for the isolation and identification of *Listeria monocytogenes* from processed meat and poultry products. In: Communication no 57. Beltsville MD.: US Department of Agriculture Food Safety Inspection Service, Laboratory; Microbiology Division; 1989.

20. Curtis GDW, Mitchell RG, King AF, Griffin EJ. A selective differential medium for the isolation of *Listeria monocytogenes. Lett Appl Microbiol* 1989; *8* : 95-8.

21. van Netten P, Perales I, van de Moosdijk A, Curtis GD, Mossel DA. Liquid and solid selective differential media for the detection and enumeration of *L. monocytogenes* and other *Listeria* spp. *Int J Food Microbiol* 1989; *8* : 299-316.

22. Jones D, Seeliger HPR. The genus *Listeria.* In: Balows A, Truper HG, Dworkin M, Harder W, Schleifer KH, editors. *The prokaryotes. A handbook on the biology of bacteria: ecophysiology, isolation, identification, applications*, Vol. II, 2nd ed. New York: Springer-Verlag; 1992 p. 1595-1616.

23. McKellar RC. Identification of the *Listeria monocytogenes* virulence factors involved in the CAMP reaction. *Lett AppI Microbiol* 1994; *18* : 79-81.

24. Dominguez Rodriguez L, Vazquez Boland JA, Fernandez Garayzabal JF, Echalecu Tranchant P, Gomez-Lucia E, Rodriguez Ferri EF, *et al.* Microplate technique to determine hemolytic activity for routine typing of *Listeria* strains. *J Clin Microbiol* 1986; *24* : 99-103.

25. Neilan BA. Identification and phylogenetic analysis of toxigenic cyanobacteria by multiplex randomly amplified polymorphic DNA PCR. *Appl Environ Microbiol* 1995; *61* : 2286-91.

26. Sambrook J, Fritsch EF, Maniatis T. Agarose gel electrophoresis. In: *Molecular cloning*: *A laboratory manual*, 2nd ed. New York: Cold Spring Harbor Laboratory; 1989 p. 6.3-6.19.

27. Wernars K, Boerlin P, Audurier A, Russell EG, Curtis GD, Herman L, *et al*. The WHO multicenter study on *Listeria monocytogenes* subtyping: random amplification of polymorphic DNA (RAPD). *Int J Food Microbiol* 1996; *32* : 325-41.

28. Wagner M, Maderner A, Brandl E. Development of a multiple primer RAPD assay as a tool for phylogenetic analysis in *Listeria* spp. strains isolated from milk product associated epidemics, sporadic cases of listeriosis and dairy environments. *Int J Food Microbiol* 1999; *52* : 29-37.

29. Yoshida T, Takeuchi M, Sato M, Hirai K. Typing *Listeria monocytogenes* by random amplified polymorphic DNA (RAPD) fingerprinting. *J Vet Med Sci* 1999; *61* : 857-60.

Reprint requests : Dr Iddya Karunasagar, Professor & Head, Department of Microbiology, University of Agricultural Sciences, College of Fisheries, Mangalore 575002, India