JOURNAL OF PURE AND APPLIED MICROBIOLOGY, October 2011. Vol. **5**(2), p. 959-962

# Identification of *Listeria monocytogenes* Isolates Based on Amplification of *hyl-A* gene

**Shakoor Ahmad Bhat* and Malik Mehboob Willayat**

Division of Veterinary Public Health, Faculty of Veterinary Sciences & Animal Husbandry
Sher-e-Kashmir University of Agricultural Sciences & Technology of Kashmir,
Shuhama, Alusteng, Srinagar - 190 006, India.

(Received: 15 February 2011; accepted: 20 March 2011)

**A total of 210 food samples (Fish-100, mutton-60, paneer-50) and 60 human clinical samples (blood-40, CSF-20) were screened for the isolation and identification of *Listeria monocytogenes* using standard microbiological technique. Seven isolates of *L. monocytogenes* were recovered, and subjected to PCR assay for detection of virulence associated with *hyl*-A genes. The isolates under study produced strong amplification of the products at approximately 456 bp, which was in agreement with the expected size confirming the isolates as *L. monocytogenes*.**

**Key words**: *Listeria monocytogenes*, Amplification, Microbiological techniques.

Listeriosis, an important bacterial zoonosis is caused by an intracellular pathogen *L. monocytogenes. Listeria* is one of most virulent food-borne pathogens with case fatality rate of 20-30% (Ramaswamy *et al.*, 2007), which can even go upto 50% in neonatal population (MacGowan *et al.*, 1991; Baron *et al*., 1994). *L. monocytogenes* is responsible for causing abortion, septicemia, meningitis, infertility, gastroenteritis and conjunctivits in both humans and animals (Aureli *et al*., 2000; Va'zquez-Boland *et al*., 2001). Food acts as a vehicle for 99% of human listeriosis cases (Mead *et al.*, 1999). During the last decade, thousands of reports have been poured showing the presence of *L. monocytogenes* in various food products including raw and ready to eat products (Bhileganokar *et al*., 1997). To monitor the incidence of *L. monocytogenes* in foods, reliable and rapid methods are need of the hour especially in the changing context. Norton (2002) has reviewed nucleic acid amplification-based methodology, specifically polymerase chain reaction (PCR)-based assays for the detection of *L. monocytogenes* in various foods and environmental samples. In the present study the isolates of *L. monocytogenes* recovered microbiologically were further confirmed by amplification of *hyl*-A gene.

## MATERIAL AND METHODS

All the seven isolates (from foods and human clinical cases) confirmed by cultural, biochemical, *in-vitro* and *in-vivo* pathogenicity test (Anton's test and monocytosis in rabbits) were screened for their virulent and non-virulent status by verifying the presence or absence of *Listeriolysin O* (*hly*-A) gene. The amplification of *hly*-A gene was carried out by following the

* To whom all correspondence should be addressed.
E-mail: bhatshakoor@gmail.com

protocol of Noterman *et al*., 1991a with slight modifications.

**DNA extraction**

Isolates of *L. monocytogenes* were grown overnight in brain heart infusion broth at 37°C. Out of this broth culture, 0.5ml culture was transformed to micro-centrifuge tube and centrifuged at 12000 rpm for 10 min. The supernatant was decanted off and pellet was resuspended in 500µl of phosphate buffer saline solution (PBSS) and re-centrifuged at 12000 rpm for 10 min. The recovered pellet was resuspended in 100µl of sterilized DNAse and RNAse free milliQ water. The pellet was suspended in the added water by vertexing and the lysis of the bacteria was performed by boiling the tube for 10 min followed by snap chilling in crushed ice. 2.5 µl of the obtained lysate was utilized as template DNA in amplification reaction volume of 25 µl.

**PCR amplification**

A pair of forward and reverse primers (Table 1) were used for amplification of 456 bp region of the *hly*-A gene in 25 µl of reaction mixture containing 2.5 µl of lysate DNA, 2.5 µl of 10X PCR buffer, 0.5 µl of 10mMdNTP, 2.0 µl of 25mM $MgCl_2$, 3 µl of 10ng/µl of the primers (forward + reverse), 0.1 µl of 5U/µl Taq DNA polymerase and 14.4 µl MilliQ water. The amplification parameters included an initial denaturation at 94°C for 2 min followed by 35 cycles each of denaturation at 94°C for 15 sec, annealing at 57°C for 30 sec, extension at 72°C for 1.30 min and a final extension at 72°C for 5 min. The PCR products were analyzed by submersive agarose gel electrophoresis to resolve the amplified DNA fragments of the target gene.

## RESULTS AND DISCUSSION

Polymerase chain reaction (PCR) has been increasingly used an important tool for the identification of *L. monocytogenes* because of its rapid, sensitive and specific detection. PCR facilitates exponential amplification of a pre-selected region of DNA and thus represents a highly specific and sensitive detection technique. PCR based assays for the detection of *L. monocytogenes* in food and environmental channels have been successfully used by many workers (Norton, 2002).).The genes of different virulent markers have also been used for the species-specific identification of *L. monocytogenes* Certain virulence genes (prfA, inlA, inlB, inlC) and virulence-associated genes (dal, LisR, clpP) intercepted with the *L. monocytogenes* genome play important role in intracellular survival, cell to cell spread and virulence of the otganism (Zhang, 2004). Recently, PCR and nested PCR for the species-specific identification of *L. monocytogenes* using primers against *hly*-A gene was standardised with a minimum detection level of 5 cells per reaction (Balamrugan, 2002). Several virulence associated genes have been sequenced and used as PCR-targets for the detection of *L. monocytogenes* e.g. *Listeriolysin O* (*hly*-A) gene (Rossen *et al.*, 1991), the *Dth 18* gene (Wernars *et al.*,1991), the *iap* A gene (Jaton *et al.*, 1992) and the *plc* A gene (Notermans *et al.*, 1991b). *Listeriolysin O* encoded by *hly*-A gene has been regarded as the most important virulence factor. In the present study, all the isolates of *L. monocytogenes* were confirmed by amplification of the *hly*-A gene. The isolates produced a strong amplification of the target gene at approximately 456 bp, thereby confirming the isolates as *L. monocytogenes* (Fig 1). In order to avoid the possible failure in the detection of virulent *L. monocytogenes*, a one step procedure enabling the demonstration of three virulence-associated genes, *prf* A, *hly* A and *plc* B simultaneously in a single PCR mixture was developed for a large scale survey (Cooray *et al.*, 1994). Recently, multiplex PCR targeting many virulence genes of *L. monocytogenes* (*plc* A, *prf* A and *hly* A genes) have been employed (Rawool,

**Table 1.** Details of the primers used for amplification of hyl A gene

| Target gene | Primer sequence | Product size (bp) | G+C content |
|---|---|---|---|
| *hyl* A Forward | 5'-GCAGTTGCAAGCGCTTGG AGTGAA-3' | 456 | 54% |
| Reverse | GCAACGTATCCTCCAGAGTGATCG-3' | | 54% |

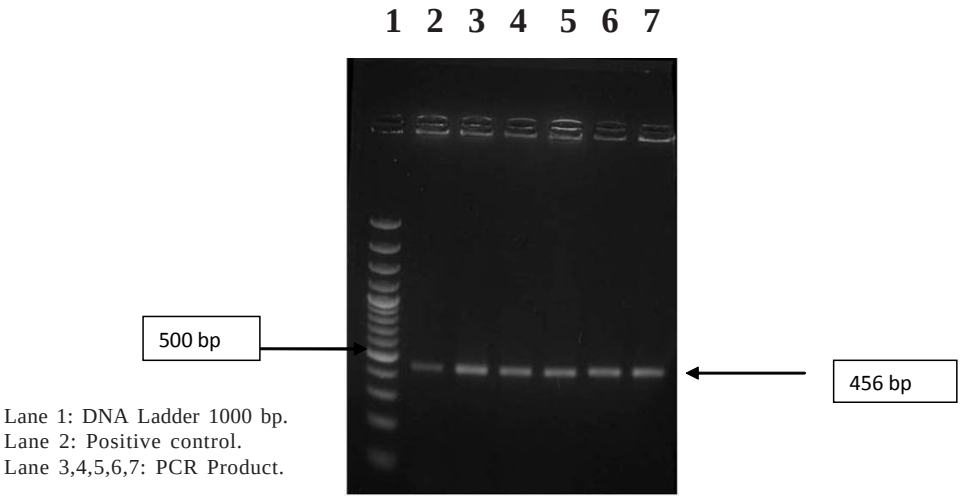


**Fig. 1.** Bands revealing amplified products of *hyl*-A gene of *Listeria monocytogenes*

2004). In conclusion, *hly*-A based PCR assay serve as an important tool for confirmation of virulent *L. monocytogenes* from human, animal, food and environmental samples giving the extract status of this important zoonotic disease.

## REFERENCES

1. Aureli, P., Fiorucci, G.C., Caroli, D., Marchiaro, G., Novara, O., Leone, L. and Salmasco, S. An outbreak of febrile gastroenteritis associate with corn contaminated with *Listeria monocytogenes*. *New England J. Med*. 2000; **342:** 1236-1241.
2. Balamurgan, 2002. Detection of *Listeria monocytogenes* from foods by polymerase chain reaction. M.V.Sc. thesis submitted to Deemed University, IVRI, Izatnagar.
3. Baron, E.J., Peterson, L.R., Finegold, S.M, Bailey, scott's. Diagonostic Microbiology. In: 9th edn, St. Louis: Mosby, 1994; pp 458-60.
4. Bhilegaonkar, K.N., Kulshreshtha, S.B., Kapoor, K.N., Kumar, A., Agarwal, R.K., Singh, B.R. Isolation of *Listeria monocytogenes* from milk. *J. Food Sci. Technol.*, 1997; **34:** 248-250.
5. Cooray, K.J., Nishibori, T., Xiong, H., Matsuyama, T., Fujita, M., Mitsuyama, M. Detection of multiple virulence-associated genes of *Listeria monocytogenes* by PCR in artificially contaminated milk samples. *App. Environ. Microbiol.*, 1994; **60(8):** 3023-3026.
6. Jaton, K., Sahli, R., Bille, J. Development of PCR assays for detection of *Listeria monocytogenes* in clinical CSF samples. *J. Clin. Microbiol.*, 1992; **30:** 1131-1136.
7. MacGowan, A.P., Cartlidge, P.H.T., Macleod, F., McLaughlin, J. Maternal listeriosis in pregnancy without fetal or neonatal infection. *J. Infect*., 1991; **22:** 53-7.
8. Mead, P.S., Slutsker, L., Dietz, V., McCaig, L.F., Bresee, J.S., Shapiro, C., Griffins, P.M., Tauxe, R.V. Food-related illness and death in the United States. *Emerg. Infect. Dis.*, 1999; **5:** 607-625.
9. Norton, D.M. Polymerase chain reaction-based methods for detection of *Listeria monocytogenes*: towards real time screening for food and environmental samples. *J. Assoc. Offic. Anal. Chem.* 2002; **85(2):** 505-515.
10. Notermans, S.H.W., Dufrenne, J., Chakraborty, T., Steinmeyer, S., Terplant, G. The chick embryo test agrees with the mouse bio-assay for assessment of the pathogenicity of *Listeria* species. *Lett. Appl. Microbiol.*,1991a; **13:** 161-164.
11. Notermans, S.H.W., Dufrenne, J., Leimeister-Wachter, M., Domann, E., Chakraborty, T. Phosphatidylinositol-specific phospholipase C activity as a marker to distinguish between pathogenic and non-pathogenic *Listeria* species. *Appl. Environ. Microbiol.*, 1991b; **57:** 2666-2670.
12. Ramaswamy, V., Cresence, V.M., Rejitha, J.S., Lekshmi, M.U., Dharsana, K.S., Prasad, S.P., Vijila, H.M. *Listeria*-review of epidemiology and pathogenesis. *J. Microbiol. Immunol. Infect.*, 2007; **40:** 4-13.
13. Rawool, D.B. 2004. Studies on rapid detection and prevalence of pathogenic *Listeria*

*monocytogenes* in bovine mastitis cases. Ph.D. Thesis, Indian Veterinary Research Institute, Izatnagar, India.

14. Rossen, L., Holmstrom, K., Olsen, J.E., Rasmussen, O.F**.** *Int. J. Food Microbiol.,* 1991**; 14:** 145.
15. Va'zquez-Boland, J.A., Kuhn, M., Berche, P., Chakraborty, T., Domi'nguez-Bernal., Wehland, J., Kreft, J. *Listeria* pathogenesis and molecular virulence determinants. *Clin. Microbiol. Rev.,* 2001; **14:** 584-640.
16. Wernars, K., Heuvelman, C.J., Chakraborty, T., Notermans, S.H.W. Evaluation of the polymerase chain reaction for detection of *Listeria monocytogenes* in soft cheese. *J. Appl. Bacteriol.,* 1991; **70:** 121-126.
17. Zhang, W., Jayarabo, B.M., Knable, S.J. Multi-virulence locus sequence typing of *Listeria monocytogenes*. *App. Environ. Microbiol*., 2004; **70:** 913-912.