J. Fish. Sci. Tech. 5(1), 54~58, 2002

JOURNAL OF
FISHERIES SCIENCE
AND TECHNOLOGY

Sequence Analysis of *iap* Gene PCR Products using *Listeria monocytogenes* Serotypes

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(Received January 2002, Accepted March 2002)

The polymerase chain reaction (PCR) amplification technique was used for comparison of *Listeria monocytogenes* serotypes. PCR primers for the fragment of invasion-associated protein (*iap*) gene were highly specific for all the serotypes of *L. monocytogenes*. Other *Listeria* spp., such as *Listeria ivanovii* and Listeria innocua were not produced the PCR fragments by above primer set. The nucleotide sequences of PCR products showed high homologies in comparison of all the isolated serotypes except unknown type II-2. The deduced amino acid sequences of the PCR products also showed similar to one another. The various region of the PCR products, called a Thr-Asn repeat region was presented. All of isolated *L. monocytogenes* serotypes possessed 16 to 20 Thr-Asn repeats.

Key words: *Listeria monocytogenes*, *iap* Gene, Serotype, PCR, Sequence analysis

Introduction

Listeria monocytogenes is a pathogen capable of causing serious illnesses, such as septicaemia and meningitis. Although listeriosis occurs infrequently with an incidence below 10 per million, the fatality rate is high, up to 75% in highly susceptible individuals such as immunocompromised individuals such as cancer, AIDS and other patients (Nφrrung et al., 1999). Currently available routine methods for the detection of *L. monocytogenes* are labour intensive and generally slow to reach a final result, requiring four days to establish presumptive presence and an additional 2~3 days to confirm and specify isolates (Farber and Peterkin, 1991). So, in the case of contamination by this organism, it is hard to protect food from poisoning and take rapid action in the scene of food processing. Thus, the polymerase chain reaction (PCR) amplification technique was recently investigated as a tool for rapid detection of *L. monocytogenes* (Manzano et al., 1998). Various specific factors involved in the virulence of *L. monocytogenes* have already been identified, such as the *hly*, the *plc*A, the *plc*B, the *inl*A, and the *prf*A genes (Datta, 1994). The sulfhydryl-activated β-hemolysin, listeriolysin O, is directly correlated with the virulence of *L. monocytogenes* (Gutekunst et al., 1992). Interest has been focused on an extracellular protein with a molecular mass of 60 kDa. This protein, termed 'p60' and encoded by the *iap* (invasion-associated protein) gene, is produced in relatively large amounts by all virulent *L. monocytogenes* strains (Kuhn and Goebel, 1989; Bubert et al., 1992; Rasmussen et al., 1995). The *iap* gene from *Listeria* spp. can be used for the development of a more versatile identification procedure for *Listeria* spp. by PCR amplication while reported PCR protocols were used mainly the listeriolysin gene for PCR (Bubert et al., 1992). The present study examined the possibility of the rapid detection method using PCR amplication of the *iap* gene of *L. monocytogenes* isolated from seafood products and seafood processing environments and investigated the characteristics between various serotypes of *L. monocytogenes* by the sequence analysis of amplified PCR products derived for *iap* gene.

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Materials and Methods

Strains

96 strains of *L. monocytogenes* isolated from imitation crab meat, *jeotgal* and frozen seafood plants were used for this study. *L. monocytogenes* KCTC 3569 was used for the standard strain. *L. innocua* ATCC 33090 and *L. ivanovii* ATCC 19119 were used for comparison strains.

Genomic DNA extraction

Culture broth (1.5 mL) was centrifuged for 10 min. at 14,000 rpm. The precipitate was treated with 750 mL of lysozyme (1 mg/mL in H_2O) for 30 min., and 8mL of proteinase K 20 mg/mL was added and further incubated at 60℃ for 1hr. Proteinase K was inactivated by heat treatment at 100℃ for 5 min., centrifuged at 14,000 rpm for 15 min., and the clear supernatant was used as genomic DNA template.

PCR amplification

MAR1 (sense) 5′-GGGCTTTATCCGTAAAATA-3′ and MAR2 (antisense) 5′-TTGGAAGAACCTTG-ATTA-3′ which amplify a 450 bp fragment of the invasion-associated protein (*iap*) gene (Manzano et al., 1998), were synthesized (Bioneer, Korea). PCR was performed in a GeneAmp PCR System 2400 (Perkin Elmer). Amplification was performed in 50 μL of reaction mixture containing 0.125 mM dNTP, 1 pmole of each primer, 1.25 U of Taq-DNA polymerase (Promega), 5 μL 10X TBE buffer, and 5 μL of template DNA. Template DNA was initially denatured at 95℃ for 5 min., followed by 35 cycles of 40 seconds denaturation at 95℃, 40 seconds primer annealing at 56℃, a 1 min. extension at 72℃, and a final extension of 7 min. at 72℃. The amplified products were detected by electrophoresis in 1.5% agarose gel in 0.5X TBE buffer.

pGEM-T Easy vector cloning

PCR products were subcloned in a pGEM-T Easy Vector System (Promega) and transformed to XL1-Blue strain. White colony was inoculated to LB broth containing ampicillin (100 μg/mL) and plasmid DNA was extracted with alkaline lysis method. Purified DNA was digested with EcoRI restriction enzyme and electrophoresed in an agarose gel with DNA size marker.

Sequence analysis

Cloned plasmid DNA was purified with a Wizard Plus SV minipreps DNA purification system (Promega). The Sequence analysis was performed using ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kits (Perkin Elmer).

Results and Discussion

L. monocytogenes belonging to all the isolated serotypes was compared with representatives of other *Listeria* spp. such as *L. innocua*, *L. ivanovii* and *L. welshimeri*. As shown in Fig. 1, only *L. monocytogenes* strains were yielded the PCR products, producing 450 bp fragment. The *iap* gene of *L. monocytogenes* encodes the major extracellular protein p60. This protein is produced in relatively large amounts by all virulent *L. monocytogenes* strains (Goebel et al., 1988). In contrast to the listeriolysin gene, the *iap* gene of *L. monocytogenes* is essential for cell viability and always detectable in the genomes of the *Listeria* spp. (Bubert et al., 1992). Thus, studies about the *iap* gene and the development of the rapid detection of *L. monocytogenes* by PCR methods are increasing. For example, in several studies, *iap*-derived primers have been applied to the species identification of *L.*

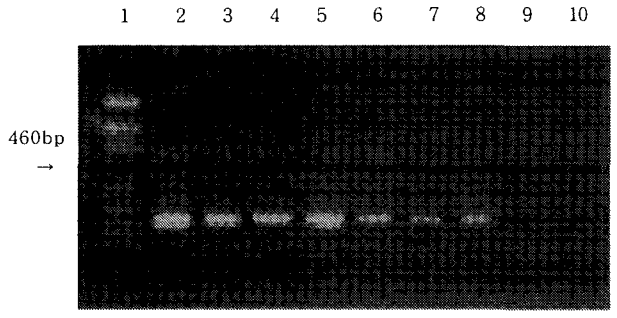

Fig. 1. Species-specific identification of *Listeria monocytogenes* by PCR.
Lanes:
1, Molecular size marker;
2, *L. monocytogenes* KCTC 3569;
3, *L. monocytogenes* Sv 1/2a;
4, *L. monocytogenes* Sv 1/2b;
5, *L. monocytogenes* Sv 1/2c;
6, *L. monocytogenes* unknown serotype I;
7, *L. monocytogenes* unknown serotype II;
8, *L. monocytogenes* unknown serotype III;
9, *L. innocua* ATCC 33090;
10, *L. ivanovii* ATCC 19119.

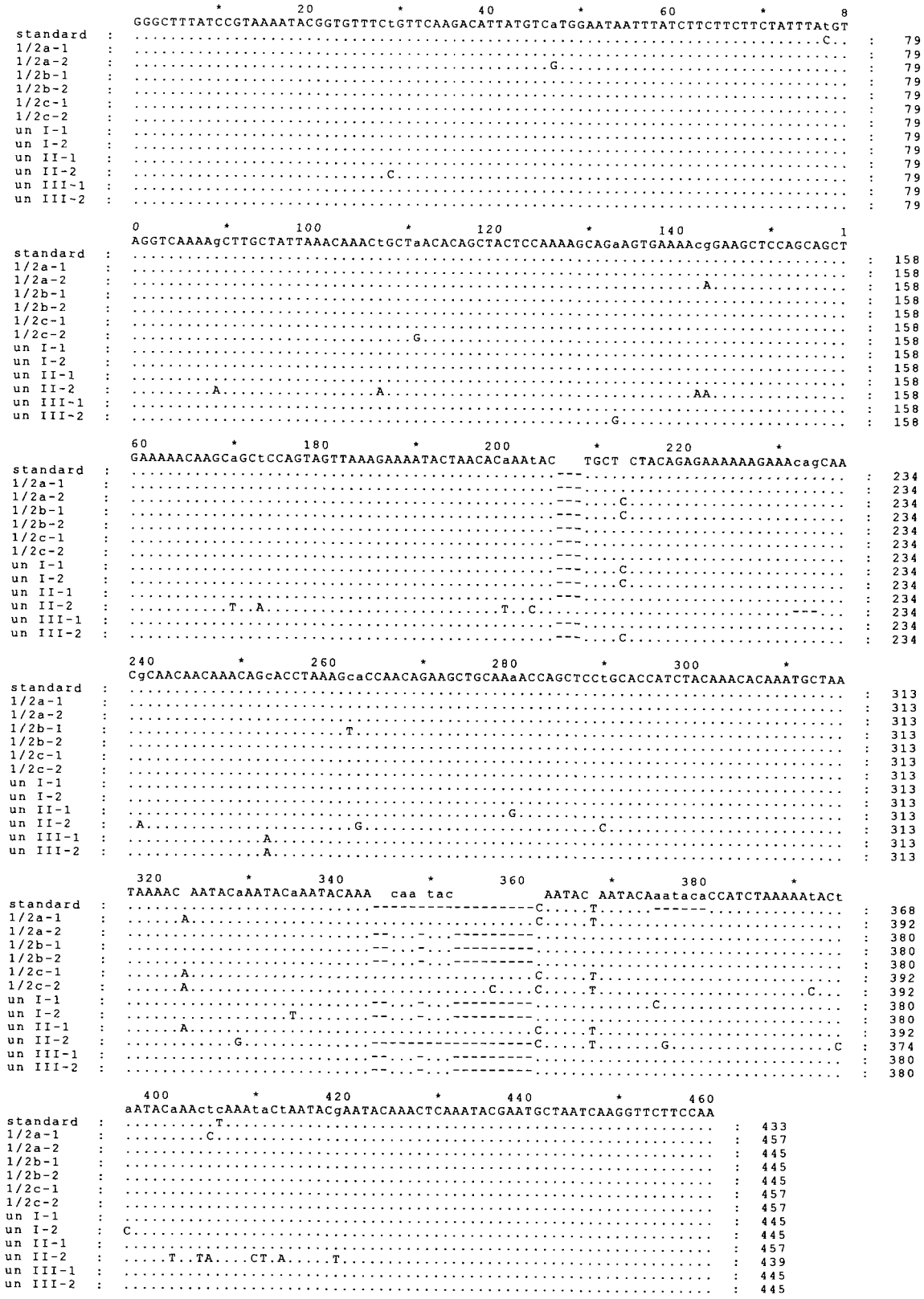

Fig. 2. Alignment of nucleotide sequence of PCR product of *Listeria monocytogenes*. The symbols refer to an identifical base (·) and an absent base (−).

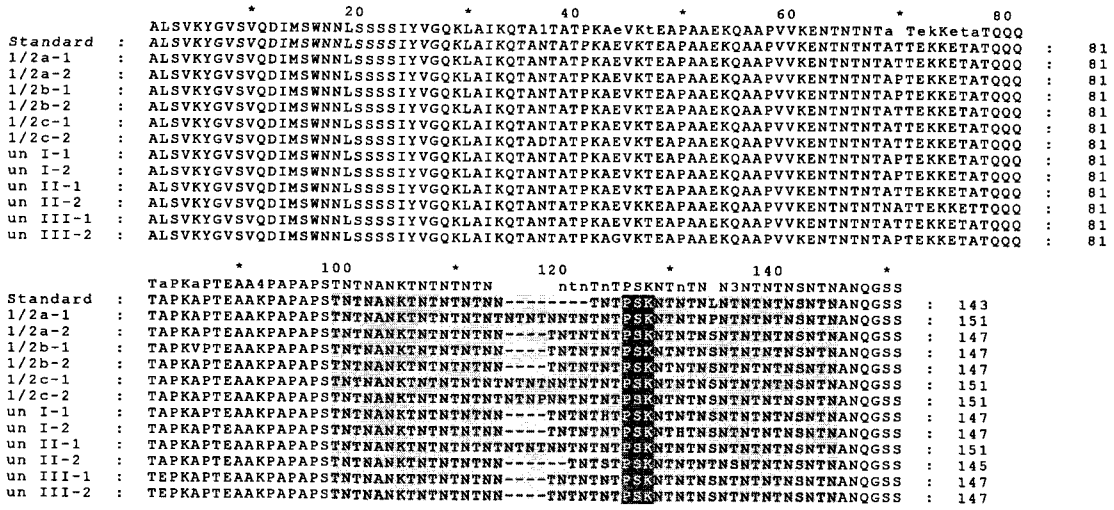

Fig. 3. Alignment of amino acid sequence of PCR product of *L. monocytogenes*. Black box indicate Thr-Asn repeat region.

monocytogenes (Allerberger et al., 1997; Bubert et al., 1997; Elsner et al., 1996). The primer and methods described in this study suggest that, on the basis of the *iap* gene are specific for *L. monocytogenes*. Therefore, the use of PCR methods for the detection of *L. monocytogens* can be an alternative to the conventional methods.

The sequence alignment of PCR products from all isolated serotypes were shown in Fig. 2. The PCR products were ranged from 433~457 bp and located within the *iap* gene. The sequence comparisons showed highly homologous among the serotypes. However, unknown type II-2 has unique sites which different from others. Forward region of fragments were highly conserved and most of various region were in the middle of fragments. The deduced amino acid sequences were shown in Fig. 3 and similar to one another. The various region, called Thr-Asn repeat region (Köhler et al., 1990) was present in all of the isolated *L. monocytogenes*. All the isolated serotypes possessed 16 to 20 Thr-Asn repeats: *L. monocytogenes* serotype 1/2a-2, 1/2 b-1 and 1/2b-2, unknown type III-1 and 2, possessed 18 repeats; serotype 1/2a-1 and 1/2c-2 possessed 19; serotype 1/2c-1 and unknown type II-1 possessed 20; unknown type I-1 and 2 possessed 17; unknown type II-2 possessed 16. The presented data show that *L. monocytogenes* isolated from seafood processing plants possessed specific Thr-Asn repeat region of *iap* gene. In addition, the strain-specific Thr-Asn repeats region may be also useful as a characteristic marker of *L. monocytogenes* studies in future research.

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